

The Role of Bile Acids in Overcoming Resistance to Chemotherapy

by

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Abstract:

In the context of cancer therapy, resistance to chemotherapy agents is a serious threat to patient welfare. In these circumstances, patients can either present with cancers that are naturally resistant to conventional therapy, referred to as innate resistance, or with cancers that become resistant following treatment, referred to as acquired resistance. In this thesis, we address the phenomenon of acquired drug resistance, involving cell lines selected for resistance to the anthracycline, doxorubicin. In the first study, we examined the role of the aldo-keto reductases AKR1C3 and AKR1B10 in doxorubicin resistance, enzymes that can hydroxylate doxorubicin to a less toxic form (doxorubicinol). Additionally, these enzymes can function to promote estrogen biosynthesis from estrone, which can have significant effects on cell growth and survival. We demonstrated in the first study that AKR1C3 and AKR1B10 are expressed at higher levels in doxorubicin resistant MCF-7 cells than their isogenic control counterparts. This change in expression correlated very well with increased estrogen synthesis. siRNA-mediated reduction in *AKR1C3* and/or *AKR1B10* transcript expression had no major effect on doxorubicin resistance, suggesting that these enzymes are not sufficient to mediate the doxorubicin resistance phenotype and that other mechanisms of doxorubicin resistance exist in these cells. We did, however, note that a pharmacological inhibitor of AKR enzymes (a bile acid termed β -cholanic acid) was effective in reversing doxorubicin resistance in doxorubicin-selected cell lines. This prompted a second study to investigate the mechanism for this reversal. We observed that β -cholanic acid strongly reduced doxorubicin resistance in cell lines that express the ABC transporter ABCC1, including doxorubicin-resistant MCF-7 breast tumour cells and H-69 lung cancer cells. Reversal of doxorubicin resistance was also observed in HEK293 cells transfected with ABCC1 expression vectors. Subsequent experiments confirmed that β -cholanic acid and another bile acid

that does not inhibit the aldo-keto reductases was able to inhibit ABCC1-mediate doxorubicin efflux from tumour cells, thereby providing a mechanism for the reversal of doxorubicin resistance. Bile acids thus represent an important new class of compounds that could prove useful in improving the effectiveness of doxorubicin chemotherapy in cancer patients, specifically in recurrent tumours overexpressing the ABCC1 transporter.

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List of Abbreviations

In Alphabetical Order

17 β -HSD	17 Beta Hydroxy-steroid Dehydrogenase
17HSD	17 Beta Hydroxy-steroid Dehydrogenase
3 α -HSD	3 Alpha Hydroxy-steroid Dehydrogenase
IC50	50% Growth Inhibition
(α/β) ₈	8 Pairs of Alternating Alpha Helices and Beta Sheets
AP-1	Activator Protein 1
AKR	Aldo-Keto Reductase
AKR1	Aldo-Keto Reductase Family 1
AKR1A1	Aldo-Keto Reductase Family 1 Sub-Family A Member 1
AKR1B1	Aldo-Keto Reductase Family 1 Sub-Family B Member 1
AKR1C	Aldo-Keto Reductase Family 1 Sub-Family C
AKR1C2	Aldo-Keto Reductase Family 1 Sub-Family C Member 2
AKR1C3	Aldo-Keto Reductase Family 1 Sub-Family C Member 3
AKR1C4	Aldo-Keto Reductase Family 1 Sub-Family C Member 4
AKR1C1-1C4	Aldo-Keto Reductase Family 1 Sub-Family C Members 1 Through 4
AKR2	Aldo-Keto Reductase Family 2
AKR6	Aldo-Keto Reductase Family 6
AKR7	Aldo-Keto Reductase Family 7
AKR7A	Aldo-Keto Reductase Family 7 Sub-Family A
AKR7A2	Aldo-Keto Reductase Family 7 Sub-Family A Member 2
ATCC	American Type Culture Collection
ARE	Anti-oxidant Response Element
BCL-xL	B Cell Lymphoma Extra Large
BCL-2	B-cell Lymphoma 2 (protein)
BCA	Bicinchoninic Acid Assay
BRCA1	Breast Cancer 1
cm	Centimeter
cDNA	Copy DNA
CCNA1	Cyclin A1 Gene
°C	Degrees Celsius
DNA	Deoxyribonucleic Acid
dNTP	Deoxyribonucleotide-Triphosphate
DC	Dextran Coated Charcoal
DC-DMEM	Dextran-Charcoal Stripped DMEM
Dox	Doxorubicin
DMEM	Dubelco's Modified Eagles Medium
EGF	Epidermal Growth Factor

Epi	Epirubicin
E2	Estradiol
ER- α	Estrogen Receptor Alpha
ESR1	Estrogen Receptor Alpha Gene
ER- β	Estrogen Receptor Beta
ESR2	Estrogen Receptor Beta Gene
ERs	Estrogen Receptors
ERE	Estrogen Response Element
E1	Estrone
EDTA	Ethylenediaminetetraacetic acid
ERK	Extracellular Regulated Kinase
FBS	Fetal Bovine Serum
G418	Geneticin
GPR-30	G-Protein Coupled Receptor 30
HRP	Horseradish Peroxidase
h	Hour
MCF-7	Human Breast Adenocarcinoma (Cell Line)
HER2	Human Epidermal Growth Factor 2
HSD11B2	Hydroxy Steroid Dehydrogenase 11 B2
Keap-1	Kelch like ECH-Associated Protein 1
kDa	Kilodalton
LB	Lysogeny Broth
MAT	MCF-7 Clones Stable Selected for AKR1C3 Transfection
MET	MCF-7 Clones Stable Selected for Empty Vector Transfection
MCF-7 _{CC}	MCF-7 Co-Culture Control Cell Line
MCF-7 _{DOX2}	MCF-7 Doxorubicin Resistant Cell Line
MCF-7 _{Epi}	MCF-7 Epirubicin Resistant Cell Line
mRNA	Messenger Ribonucleic Acid
μ m	Micrometer
μ g	Microgram
μ l	Microliter
mM	Milimolar
mg	Milligram
ml	Milliliter
min	Minute
MAPK	Mitogen Activated Protein Kinase
M	Molar concentration
M-MLV	Murine Leukemia Virus
nm	Nanometer
nM	Nanomolar

NADPH	Nicotinamide Adenine Dinucleotide Phosphate
Nrf2	Nuclear Factor (erythroid-derived 2) Like 2
ORF	Open Reading Frame
%	Percentage
PBS	Phosphate Buffered Saline
PI3K	Phosphatidylinositol 3-Kinase
PCR	Polymerase Chain Reaction
Q-PCR	Quantitative Polymerase Chain Reaction
RIPA	Radio Immuno Precipitation Assay
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
RNA	Ribonucleic Acid
sec	Second
SERM	Selective Estrogen Receptor Modulator
s118	Serine Residue 118
AKT	Serine/Threonine Protein Kinase
x g	Times g Force
TBS	Tris-Buffered Saline
TBST	Tris-Buffered Saline Plus Tween 20
TMOD1	Tropomodulin 1
TNF- α	Tumour Necrosis Factor Alpha
UV	Ultraviolet
v/v	Volume per Volume
w/v	Weight per Volume

List of Publications

- Edwardson, DE, Chewchuk, S, Parissenti, AM, (2012): **Resistance to Anthracyclines and Taxanes in Breast Cancer**. Breast Cancer Metastasis and Drug Resistance, Chapter 13, pp 227-247.
- Edwardson, DW, Narendrula, R, Chewchuk, S, Mispel-Beyer, K, Mapletoft, J., Parissenti, AM, (2015): **Role of Drug Metabolism in the Cytotoxicity and Clinical Efficacy of Anthracyclines**. Current Drug Metabolism, 16 (6): pp 416-426.
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Chapter 1

Introduction

1.1: Cancer

Cancer is a medical condition in which cells of the body begin to proliferate abnormally in a highly invasive, unregulated capacity (1, 2). These cells can be freely circulating as in leukemias, or can form solid tumors as in breast or lung cancers. It should be noted that cancer can originate from and affect every tissue type in the body. In general, cancer begins as benign growths which undergo transformation into malignancies. These malignancies are characterized by 6 known hallmarks as defined by Hanahan and Weinberg in 2000 and later revised in 2011 (1). Briefly, these hallmarks are listed as: sustained proliferative signalling, evading growth suppression, resisting cell death, enabling replicative immortality, inducing angiogenesis and activating invasion and metastasis. Current treatments for cancer include surgery to remove tumorous growths, radiation and /or chemotherapy to treat non-solid cancers or, in the case of solid tumors, to eliminate any remaining cancerous cells (2).

Cancer is a condition that will occur in roughly 45% of Canadian women and 49% of Canadian men according to the statistics presented by the Cancer Society of Canada in 2017 (3). Current estimates are that 1 in 4 Canadians will succumb to this disease. Cancer is characterized by abnormal growth and invasion of cells into neighbouring tissues. This may also include the spreading of the cancerous cells away from the primary tumour site to distant tissues through the blood stream or lymphatic system known as metastasis. The causes of this disorder are generally attributed to either environmental factors or hereditary factors or some combination of the two (4–9). Among the identified environmental contributors to cancer incidence are exposure to: tobacco smoke, infections (particularly viral), radiation exposure and environmental pollutants

(3). Cancer is often initially detected by the appearance of local symptoms which include lumpy growths (tumours), hemorrhage, ulceration, and pain. Symptoms of metastasis include enlarged lymph nodes, enlarged liver or spleen, pain, fracture of bones that are affected, as well as neurological symptoms. In some cases, systemic symptoms develop distant from either the primary tumour or the subsequent metastases. These include weight loss, either from lack of appetite or cachexia, fatigue, excessive sweating, anemia, as well as other paraneoplastic phenomena (3). Due to the complex nature of this disease, finding successful treatments has proven difficult. In this light, much emphasis has been placed on cancer prevention and early detection or screening of tumours.

The most common cancers diagnosed in North America affect lung, prostate, breast and colorectal tissues, which account for roughly 50% of new cancer cases diagnosed every year. Lung cancer accounts for nearly 26% of all deaths caused by cancers in either men or women (3). Breast cancer accounts for 13.1% of cancer deaths in women and prostate cancer accounts for 9.6% of cancer deaths in men, with colorectal cancer deaths now reaching 12% in men and 11.3% in women (3). While the rates of cancer incidence have been steadily on the rise, we can now boast an overall survival rate from cancer at 60%, with some cancers such as testicular or thyroid cancers having a survival rate as high as 96% and 98%, respectively (3). Unfortunately not all cancer types can share this impressive statistic. Cancers of the esophagus or pancreas tend to have very low survival rates, on the order of 14% and 8%, respectively (3). Survival statistics are highly dependent on several issues which include but are not limited to: tissue or origin, availability of early detection technologies, and treatment options, which are often dependent on tumour type, subtype and stage.

1.2: Breast Cancer

Breast cancer can be categorized into 2 broad types: invasive and non-invasive carcinomas (10).

1.2.1: Non-Invasive Breast Cancer

The non-invasive breast cancers generally present as one of two types: ductal carcinoma *in situ*, and lobular carcinoma *in situ* (11, 12). Within these types, each cancer can then be “staged” and “graded” based on the level of tumor growth spread and the level of differentiation observed in the cells, respectively. Briefly, stage 0 are low grade cancers that tend to be highly differentiated and highly localized with a very high rate of treatment success. As the grades and stages increase in number, cells become less differentiated as compared to the normal cells of the related tissue, and tend to become more invasive leading to an increased risk of metastasis (stage 4 are high grade and describe a poorly differentiated metastatic cancer) (10). It should be noted that the definition of *in situ* specifically refers to a cancerous lesion which remains at its site of origin and is therefore non-invasive (11–13). While these *in situ* carcinomas can be found at all stages, from low to high grade tumors, many are now detected at early stages thanks to improved screening techniques. These screening techniques also account for overall increases in diagnosis of both lobular and ductal *in situ* carcinoma (14, 15). Ductal *in situ* carcinoma has historically been diagnosed more frequently than lobular carcinoma *in situ*, accounting for nearly 70% of non-invasive breast carcinomas (11). Lobular carcinoma *in situ* is currently regarded as a marker during the development of invasive lobular carcinoma (15). Each of these *in situ* carcinomas has historically been treated in the same manner with radical surgery being preferred (11, 12, 14–16). As a result, little data exists on the true progression of these *in situ* carcinomas. Recent studies have attempted to derive data on the progression of these cancers to better tailor

treatment for patients. While preferred treatment for lobular carcinoma *in situ* continues to be radical mastectomy, it is becoming more common in modern medicine to treat with breast sparing surgery followed by chemo- or radiotherapy (11, 15). Ductal carcinoma *in situ* is also treated with breast sparing methods followed by chemo- or radio therapy to prevent recurrence with reasonable success (11, 14, 15).

1.2.2: Invasive Breast Cancer

Little is known about the disease progression of lobular carcinoma *in situ* as it is a relatively rare form of breast cancer, is usually detected early, and is typically treated rapidly and radically with good success overall (12, 15). However, it is known that lobular carcinoma can become invasive. Invasive lobular carcinoma has a relatively low occurrence rate, accounting for approximately 10% of all breast cancers (17). Invasive lobular carcinoma possesses a unique growth pattern wherein strands of single cells tend to spread away from the tumour origin (18, 19). Due to the unique properties of their growth, central tumours of invasive lobular cancer tend to not form distinct masses and can be difficult to distinguish from surrounding tissues using conventional means (17, 19). Early diagnosis of invasive lobular carcinoma is very difficult as a result of this and thus tends to be detected later in life (18). Various studies have been done to compare differences in patient outcomes between invasive lobular and invasive ductal carcinomas with mixed results (17). A 1996 study showed while patterns of metastasis and spread were different between invasive lobular and invasive ductal carcinomas, no statistical difference in overall survival could be detected (20). A contrasting study from 2005 indicated that following treatment by conventional chemotherapy (anthracycline and/or taxane as primary chemotherapy), patients with invasive lobular carcinoma were less likely to have a pathological

complete response than those with invasive ductal carcinoma, however they did exhibit a longer recurrence free survival rate than their counterparts with invasive ductal carcinoma (21).

The most common form of invasive breast cancer is invasive ductal carcinoma accounting for 80% of all invasive breast cancers (10). As a result, invasive ductal carcinoma tends to define a much broader spectrum of disease than invasive lobular carcinoma. Aside from their obvious histological differences, invasive ductal carcinomas tend to present as malignancies more often, albeit not exclusively (22–24). In addition, upon genetic/molecular analysis of each histologic type, significant differences can be observed (22). While lobular and ductal cancers comprise the majority of breast cancers, invasive or otherwise, other specific types exist based on histology. Regardless of the type of breast cancer almost all are further subdivided into types based on various genetic markers. These include steroid and growth receptors (estrogen receptor, progesterone receptor, HER2 receptor) (25), and the presence of absence of BRCA1 mutations as the most common markers (25).

1.3: Treatment of Breast Cancer

Treatment of patients for breast cancers can take several forms depending on the type, grade and stage of their cancer. Surgery is historically preferred as this procedure will typically remove the disease-causing cells from the body before metastasis occurs (26). While surgery is an adequate treatment option for certain cancers, in many (if not all) cases it is accompanied or followed by chemotherapy or radiation therapy, depending on the type and stage of the cancer (26). Ionizing radiation is used prior to surgery to shrink tumors to facilitate their subsequent removal. In most cases of breast cancer, radiation is typically used post-surgery to ensure complete destruction of the tumour, including local metastases (26). In other cases, chemotherapy drugs are used in the same capacity, depending again on the specific cancer

presented. In the specific case of chemotherapy drugs, they can be used prior to surgery (known as neo-adjuvant chemotherapy) (26), to shrink the tumours and provide better margins for surgical removal, or can be applied following surgery (known as adjuvant chemotherapy) to ensure that no cancer cells are left (26). It should be noted at this time that preferences for adjuvant vs. neoadjuvant chemotherapy differ based on global regions (27). In North America, for instance, while neoadjuvant chemotherapy is performed, adjuvant chemotherapy is preferred. Several European nations have adopted the opposite policy preferring neoadjuvant therapy. A study from 2005 comparing cohorts of patients who underwent adjuvant chemotherapy showed lower recurrence than those who underwent neoadjuvant chemotherapy (26). Conversely, neoadjuvant chemotherapy has been demonstrated to reduce tumour size offering improved survival and in some cases to eliminate cancerous cells without the need for surgery (27). Specific chemotherapy regimens for breast cancer will be discussed in Chapter 2 of this thesis in more detail. While chemotherapy agents have proven somewhat effective in treating cancers, they present several drawbacks. Chief among the weaknesses of chemotherapy drugs are their broad range of adverse side effects (29). These side effects vary depending on the chemotherapy agent used, but typically include: nausea, vomiting, neutropenia, hepatotoxicity, renal toxicity, cardiotoxicity, cachexia (muscle wasting), and in some cases neurotoxicity (28, 29).

1.4: Anthracyclines

Anthracyclines are a class of chemotherapeutic drugs derived from *Streptomyces peucetius* and are among the most effective chemotherapeutic agents developed to date (30). This is partly due to the wide range of cancer types that can be successfully treated with this class of drugs, including leukemias, lymphomas, and tumours of the breast, uterus, ovary, and lung (31). The first anthracycline discovered was daunorubicin (31). It was found that

daunorubicin possessed positive anti-tumour activity in murine models (31). Clinical trials began in the 1960's and proved successful in treating patients with leukemias, but had serious cardio toxic side-effects (31, 32). For this reason, researchers sought to modify the structure of daunorubicin in the hope of finding less toxic, but equally effective, analogues of the drug. To this end, doxorubicin and epirubicin were developed (31). Doxorubicin shares a common scaffold structure with daunorubicin, but has an additional hydroxyl group (31). Epirubicin has a similar structure to doxorubicin, however the hydroxyl group on the 4' carbon of the sugar group has a different stereochemistry. Both doxorubicin and epirubicin are favoured for use as chemotherapy drugs over daunorubicin; however, epirubicin is favored over doxorubicin for cancer treatment as it has lower cardiotoxicity with only a small loss in efficacy (32, 33).

Specific actions of anthracyclines will be discussed in detail in chapter 2. Briefly, they function by intercalating DNA, interfering with topoisomerase II, and generation of iron-mediated oxygen-derived free radicals (31, 33, 34). Since tumour cells often divide more rapidly than most cell types within the body (except hematopoietic or intestinal crypt cells), the above mechanisms likely account for the anti-tumour properties of anthracyclines. Despite the success of these agents as chemotherapeutic drugs, their use is not without risk. Common acute side effects include nausea, vomiting, heart arrhythmias, neutropenia, and alopecia (31, 34). Chronic side effects, as a result of high cumulative dosage, can result in chronic heart failure, dilated cardiomyopathy, and death (31, 34). These side effects are most strongly associated with doxorubicin treatment; it is for these reasons that epirubicin is preferred as a chemotherapeutic agent (31, 34, 35). While anthracyclines are used as primary chemotherapy in breast cancer, they are by no means an exclusive treatment method. Several other chemotherapy agents are briefly discussed as part of common treatment regimens in Chapter 2 of this thesis. It should be

noted at this time that while anthracyclines have common defined side effects, others may occur depending on the specific combination of chemotherapy agents used (28, 29).

1.5: Chemotherapy Resistance in Breast Cancer

These toxic side effects are further complicated by the occurrence of chemotherapy resistance, a phenomenon which is observed in all cancer types and subtypes. The degree of resistance also varies depending on disease type and treatment applied. Briefly, resistance to chemotherapy has been observed to occur via two different scenarios. First, some cancers exhibit innate resistance to chemotherapy and do not respond to initial treatment (often referred to as “primary or innate chemotherapy resistance”). These cancers are usually treated with stronger chemotherapy drugs, if possible, or alternate treatments may become necessary, which may include surgery or radiation therapy (36). A second form of resistant cancers has also been described in patients that undergo a full course of chemotherapy. Patients will initially either respond or show partial response to the therapy. In many cases, a fraction, or the majority, of the tumour cell population is killed (36, 37). The remaining cells survive and continue to replicate, resulting in disease progression. When the patient returns for treatment, the tumour is no longer responsive to the previously used chemotherapy agent and is termed drug resistant. In addition, these tumours will frequently not respond to a wide variety of chemotherapeutic agents, resulting in a phenomenon known as multi-drug resistance (38, 39).

To further complicate our understanding of chemotherapy resistance, differences in resistance pathways are often observed between *in vitro* and *in vivo* systems. This leads to a critical weakness in studying chemotherapy resistance, in that most studies conducted on chemotherapy resistance are performed using *in vitro* systems (41–43). As we will discuss in more detail in Chapter 2 of this thesis, we will see that in many circumstances the mechanisms of

resistance identified in these *in vitro* systems may not translate to *in vivo* systems. The best example of this is related to the drug transporter ABCB1, also known as the multidrug resistance protein 1 (MDR1) or P-glycoprotein (P-gp) (41). ABCB1 has been shown to be expressed at high levels in a variety of *in vitro* cancer cell lines selected for resistance to the most commonly used chemotherapy agents, which include the various anthracyclines and taxanes as well as several other drugs (40). This increased expression has been shown to be critical for the *in vitro* resistance to chemotherapy (41, 42). Effective chemical inhibitors for ABCB1 exist and have been assessed in clinical trials, but the usage of these inhibitors is often associated with increased neurotoxicity as ABCB1 is a key xenobiotic defence mechanism in the blood-brain barrier and are therefore not very useful clinically (42). We provide this example to show that while *in vitro* studies are important in developing insight into new approaches to treat chemotherapy-resistant cancers, they do not often tell the whole story and may not translate well into a clinical setting (42). It should also be noted that setbacks such as these are why we must pursue further studies into understanding clinically-relevant mechanisms of chemotherapy resistance.

Various other mechanisms associated with chemotherapy resistance will be discussed in greater detail in Chapters 2 and 3 and include: increased expression of metabolic enzymes to detoxify chemotherapy agents, reduced expression of pro-apoptotic proteins to prevent cell death and enhance survival, increased expression of cell survival proteins, and changes in expression of chemotherapy target proteins (41, 42). For the purpose of this study we focussed on the mechanisms of resistance associated with anthracycline resistance, specifically doxorubicin.

In the following chapters we will discuss the role of 2 major resistance mechanisms with specific hypotheses and aims discussed in Chapters 4 and 6 of this thesis. First, we will examine in detail the various treatment regimens used in breast cancer treatment and the resistance

mechanisms associated with anthracyclines as well as a second class of drugs called taxanes. We will then focus on anthracycline resistance and the role of chemotherapy metabolism in this phenomenon, in particular on the doxorubicin-inactivating enzymes known as the aldo-keto reductases (AKRs). We will show that while these enzymes can play a role in chemotherapy resistance, ABC transporters, specifically ABCC1, play the dominant role in MCF-7 breast cancer cells selected for doxorubicin resistance *in vitro*. Interestingly, we will present data that shows the efficacy of using a bile acid, β -cholanic acid (normally used as an inhibitor of AKRs), to sensitize certain doxorubicin-resistant cell lines to doxorubicin treatment. Finally, we will show that this sensitization is related to the expression of the ABCC1 transporter and that cells which express ABCB1 are unaffected by treatment with bile acids. Our aim is to demonstrate the utility of a compound which is capable of inhibiting multiple mechanisms of chemotherapy resistance, and this could have a great impact on improving patient care in the future.

1.6: Hypotheses and Specific Aims

I postulate that the increase in AKR1C3 expression will result in higher levels of serum estradiol. This will result in one of two possibilities: 1) An increase in estrogen signaling will resulting in enhanced survival and increased growth of the cells, or 2) increased estradiol levels that will result in a negative feedback in turn reducing the ER α levels and resulting in a down regulation of the growth-promoting signal. We also hypothesize that in addition to their role in modulating AKR1C3 function, bile acids also have a regulatory role in ABCC1-mediated drug transport. Based on the above hypotheses, the primary aims of this thesis are to:

- 1) Assess AKR1C3 protein levels in wildtype and anthracycline-resistant MCF-7 breast tumour cells,
- 2) Determine if overexpressing AKR1C3 in wildtype MCF-7 cells impart resistance to anthracyclines,
- 3) Use siRNA approaches in MCF-7_{DOX2-12} cells to reduce levels of AKR1C3 and AKR1B10 and determine the effect on resistance to doxorubicin
- 4) Assess the ability to convert estrone to estradiol in MCF-7 anthracycline-resistant and AKR1C3-transfected cells,
- 5) Assess the estrogen receptor signaling activity in MCF-7 Anthracycline-resistant cells by assessing expression levels of BCL-2 and Cyclin D1 as indicators of genomic signaling,
- 6) Assess the growth rate of MCF-7 Anthracycline resistant and AKR1C3 transfected cells to confirm the reduced rate of growth in anthracycline resistant cells,

- 7) Monitor chemotherapy resistance in a variety of ABCC1 expressing cell lines including the MCF-7_{DOX2-12} cells, H69_{AR} (doxorubicin resistance small cell lung carcinoma) cells, and HEK293_{MRP1} transfected with ABCC1.
- 8) Monitor intracellular accumulation of doxorubicin in the aforementioned cells by flow cytometry.
- 9) Verify that ABCC1 is being inhibited by monitoring glutathione levels intracellularly.
- 10) Determine if the inhibition of chemotherapy transport is specific to ABCC1 or if ABCB1 can also be affected by bile acid treatment.
- 11) Determine the nature of β -cholanic acid-mediated ABCC1 inhibition.

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Chapter 2

Resistance to Anthracyclines and Taxanes in Breast Cancer

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2.1: Contributions by Authors:

SC authored the introduction and sections pertaining to anthracycline resistance and the management of patients with resistant tumors. DE authored sections pertaining to taxane resistance and *in vivo* taxane and anthracycline resistance. DE was also the primary author of the manuscript. SC and DE had equal contribution to sections regarding tumor initiating cells. AMP participated in concept and organization of the manuscript, assisted in editing and drafting of the manuscript and authored the abstract and concluding remarks. All authors read and approved the manuscript.

2.2: Abstract

Taxanes and anthracyclines are widely used in chemotherapy regimens for the treatment of invasive breast cancer. Whether used in the neoadjuvant or adjuvant settings, numerous clinical trials have validated their effectiveness in improving both progression-free and overall survival in breast cancer patients. However, while clinical response (decrease in tumor size by palpation) is common, for many patients this response is short-lived, after which tumors become refractory to treatment. In addition, some tumors exhibit innate (intrinsic) resistance to these regimens at the start of treatment. Consequently, the vast majority of patients do not exhibit either a pathologic complete response post-treatment or a survival benefit from chemotherapy. Numerous *in vitro* studies have identified potential mechanisms of action for the anthracyclines and taxanes and how tumors may evade the cytotoxic properties of these agents, but their clinical relevance remains questionable. *In vivo* studies of drug resistance are less subject to such criticisms, but false discovery rates can be high, in particular for genomic studies of biomarkers of drug response or resistance. Nevertheless, studies of drug response and resistance are now starting to provide useful tools to distinguish between responding and non-responding tumors and insight on how to best treat patients with tumors that are refractory to treatment.

Keywords: Incidence rates, Adjuvant therapy, Estrogen Receptor (ER) positive cancers, Taxanes, Anthracyclines, *In vitro* studies, Drug transporters, Aldo–keto reductase enzymes (AKRs), Microtubules, β -tubulin isotypes, Apoptosis, Chemotherapy resistance, Stromal cells, Tumor initiating cells (TICs), Chloroquine

2.3: Introduction

Breast cancer is the most common form of cancer among women worldwide, with incidence frequencies continuing to rise (1). This increasing incidence is generally attributed to prolonged life expectancy, urbanization and adoption of western lifestyles (1). Global statistics as of 2004 from the World Health Organization (WHO) estimate that breast cancer comprises roughly 16 % of all female cancers worldwide (1); of these, an estimated 519,000 women succumbed to the disease in 2004 alone. The WHO estimates that a majority of these deaths occurred in developing countries, roughly 69 % (1). While incidence rates vary greatly worldwide they have been recorded to be as high as 99.4 per 100,000 women in North America. Moderate incidence rates have been recorded in Eastern Europe, southern Africa, eastern Asia and South America, with the lowest incidence rates occurring in most African countries (1, 2). As is the case with incidence rates, survival rates also vary greatly worldwide, ranging from 80 % in high income nations to less than 40 % in low income nations. These discrepancies are mostly attributed to availability of early detection and treatment methods (1, 2).

Upon detection of disease that is contained within the breast, the primary treatment for breast cancer is typically surgical resection of the tumor with negative margins to prevent recurrence (3). This is because many patients with early-stage disease respond well to this treatment method. If the disease is sufficiently advanced but within the axilla, many adjuvant treatments exist for breast cancer which include radiation therapy and a variety of chemotherapy regimens (3). Adjuvant therapy is generally designed to treat micro metastatic disease or breast cancer cells that have escaped the primary tumor but not yet established identifiable metastases. Specific treatments differ depending on the nature of the tumor subtype (3). Locally advanced

and inflammatory breast cancers, however, do not respond well to primary surgical techniques and are therefore deemed inoperable. Neoadjuvant chemotherapy regimens are thus used as the first treatment for these breast cancers and typically include the anthracyclines and taxanes. These regimens include but are not limited to: TAC (Taxotere (Docetaxel), Adriamycin (Doxorubicin), and Cyclophosphamide) (4, 5), AC T (Adriamycin and Cyclophosphamide followed by Taxol) in both conventional and dose-dense regimens (6, 7), FEC 100 (5-fluorouracil, Epirubicin, Cyclophosphamide) (8), FAC (5-fluorouracil, Adriamycin, Cyclophosphamide) (9, 10), TC (Taxotere, Cyclophosphamide), or TCH (Taxotere, Carboplatin, and Trastuzumab (Herceptin) for HER2-positive tumors (11) (see **Table 2-1**, adapted from WebMD <http://emedicine.medscape.com/article/1946040-overview#aw2aab6b3>). Each of these chemotherapy drugs serves a different function in treatment. The taxanes (paclitaxel and docetaxel) function as anti-microtubule agents disrupting the cell's ability to divide during mitosis (4, 5). The anthracyclines (doxorubicin and epirubicin) function as DNA-damaging antibiotics (6, 7).

Cyclophosphamide is an alkylating agent, adding alkyl groups to the guanine bases of DNA, and Trastuzumab is a monoclonal antibody targeting and inhibiting the HER2 growth receptor present on some breast cancer types (9, 10). Additionally, in early-stage breast cancer, adjuvant chemotherapy can play a critical role in the treatment of Estrogen Receptor (ER) positive cancers (12). Adjuvant therapy in these cases involves the use of compounds that target the estrogen signaling pathway, either through interfering with estrogen synthesis (aromatase inhibitors (Letrozole) or through selective estrogen receptor modulators (SERMs (tamoxifen)) (13).

Table 2-1 Anthracycline and taxane containing regimens for the treatment of breast cancer

Treatment Regimen	Chemotherapy agents used	Dose	Frequency	Cycles	Reference
TAC	Taxotere (Docetaxel) Adriamycin (Doxorubicin) Cyclophosphamide	75 mg/m ² IV 50 mg/m ² IV 500 mg/m ² IV	Every 21 days	6	(4,5)
AC→T	Adriamycin Cyclophosphamide Followed by Taxol (Paclitaxel)	60 mg/m ² IV 600 mg/m ² IV 175 mg/m ² IV	Every 21 days (14 days for dose dense) Every 21 days (14 days for dose dense)	4 4	(6,7)
FEC 100	5-Fluorouracil Epirubicin Cyclophosphamide	500 mg/m ² IV 100 mg/m ² IV 500 mg/m ² IV	Every 21 days	6	(8)
FAC	5-Fluorouracil Adriamycin Cyclophosphamide	600 mg/m ² IV 60 mg/m ² IV 600 mg/m ²	Every 21 days	4	(9,10)
TC	Taxotere Cyclophosphamide	75 mg/m ² IV 600 mg/m ² IV	Every 21 days	4	(11)
TCH	Taxotere Carboplatin Trastuzumab (Herceptin)	75 mg/m ² IV AUC 6, IV 4 mg/kg loading dose IV followed by 2 mg/kg/wk X 18 then q3wk X 12	Every 21 days	6	(11)

Even with such available treatments, disease progression typically occurs in advanced breast cancers, likely due to the presence or development of chemotherapy-resistant tumors (14). Some patients possess tumors that exhibit innate resistance to chemotherapy and do not respond to initial treatment (often referred to as “primary chemotherapy”). These cancers are then typically treated with other chemotherapy drugs, if possible, or alternate treatments may become necessary, including surgery or radiation therapy (15). Other patients have tumors that initially respond or show partial response to the therapy. In such cases, a fraction or even the majority of the tumor cell population is killed (15, 16). The remaining drug-resistant cells, however, survive and continue to replicate, resulting in disease progression. Here we will explore some of the mechanisms associated with resistance to taxanes and anthracyclines in the treatment of breast cancers as well as some of the current work being done to manage patients with drug-resistant tumors.

2.4: Resistance to Anthracyclines and Anthracycline-Based Regimens *In vitro*

Anthracyclines are believed to be cytotoxic to tumor cells through three mechanisms. First, they intercalate between strands of DNA or RNA molecules and interfere with normal synthesis of these macromolecules in rapidly dividing cells (17). Second, they interfere with topoisomerase II, which is normally responsible for relaxing supercoiled DNA in order to facilitate DNA replication and transcription (18). Finally, anthracyclines cause cellular damage by facilitating the creation of iron-mediated oxygen free radicals (18).

Many of the biochemical and cellular mechanisms of anthracycline resistance that have been identified to date have been obtained from *in vitro* studies.

2.4.1: Drug Transporters and Anthracyclines

The innate or acquired overexpression of drug transporters has been proposed as a possible mechanism of resistance to anthracyclines in breast cancer and has been observed primarily in cells exposed to high concentrations of these agents (19–21). The drug transporters are typically integral “ATP-binding cassette” (ABC) membrane proteins that actively transport anthracyclines and other chemotherapy drugs from tumor cells in an ATP-dependent manner (19–21). By exporting drugs from the cytoplasm into the extracellular space, tumor cells are protected from the damaging effects of the chemotherapy agents (19–21). As revealed in a recent study (15), selection of tumor cells for survival in increasing concentrations of anthracyclines resulted in the acquisition of anthracycline resistance at a specific threshold dose. At or above this threshold dose, uptake of anthracyclines was substantially reduced. Coincident with the acquisition of drug resistance and reduced drug uptake into tumor cells was the increased expression of various ABC transporters, specifically Abcb1, Abcc1, and Abcc2 (15). The induced ABC transporter differed depending on the cell line examined: for example, epirubicin resistant cells showed elevated levels of Abcb1 when the selection dose reached a 30 nM concentration, while doxorubicin-resistant cells only showed elevated levels of Abcc1 late during selection (100 nM doxorubicin) (15). While the expression of these transporters correlated well with the reduced cellular uptake of drugs, their expression did not correlate well with drug sensitivity, suggesting that multiple factors were at play in the acquisition of drug resistance (15).

2.4.2: Alterations in Anthracycline Metabolism

An additional mechanism for anthracycline resistance appears to involve the ability of the liver and possibly breast tumor cells to convert chemotherapy agents into considerably less

cytotoxic forms (22), thus protecting tumor cells from the killing action of these agents. One example of this is moderated by the overexpression of the aldo–keto reductase superfamily of enzymes (AKRs) (16). The AKRs reduce ketones and aldehydes into secondary and primary alcohols (23) and their expression has been shown to be regulated by osmotic pressure, AP-1 transcription factors, and anthracycline-generated reactive oxygen species (ROS). The AKR1C family of enzymes has been shown to metabolize a variety of chemotherapy agents, including doxorubicin (23). AKR1A1 and AKR1C2 have been shown to convert the anti-tumor agent doxorubicin into doxorubicinol, a significantly less toxic anthracycline (23). In a similar fashion to AKRs, carbonyl reductases and quinone oxidoreductase-1 (NQO1) have been shown to metabolize doxorubicin into doxorubicinol (24). The conversion of doxorubicin to doxorubicinol appears to result in altered localization of the drug to lysosomes (16), such that the drug no longer reaches its target in the nucleus.

2.4.3: Other Putative Anthracycline Resistance Mechanisms *In vitro*

In addition to the expression of drug efflux pumps and drug metabolizing enzymes, other proteins have been implicated in anthracycline resistance *in vitro*, including the downregulation of topoisomerase II (25), changes in p53 function (26), and reduced drug-induced apoptosis (27). Gene expression profiling studies suggest that a variety of genes change expression as breast tumors acquire resistance to anthracyclines (27). It remains unclear how many of these genes play a *bona fide* role in clinical resistance to anthracyclines and how many are “passenger” genes that change expression with the “drivers” of drug resistance.

2.5: Mechanisms of Resistance to Taxanes *In vitro*

The taxanes block the growth of tumor cells by binding to microtubules and preventing their depolymerisation, leading to mitotic catastrophe (28), multinucleation of cells, and the induction of apoptosis (29). One such apoptosis-inducing agent upregulated by the taxanes is the cytokine TNF α (30). Like other chemotherapy drugs, the efficacy of taxane treatment is limited by a tumors' inherent or acquired ability to resist their killing action. Taxane resistance can be the product of a variety of alterations in cell behavior (29). A number of potential mechanisms for taxane resistance have been identified *in vitro*, including elevated expression of the ABC family of drug transporters, alterations in microtubule structure and stability, inhibition of apoptosis, as well as the activation of some survival pathways.

2.5.1: Drug Transporters and Taxanes

One of the most studied mechanisms of drug resistance is the overexpression of the ABC transporters (31). ABC transporters are highly expressed in some tissues such as the intestinal epithelium and in less differentiated cell types (32). They are associated with the membrane and actively transport a variety of molecules out of the cell (31). Among the ABC transporters is the permeability glycoprotein 1 (P-gp), also known as multi-drug resistance protein 1 (Mdr1) or Abcb1. It has been shown that P-gp contributes to taxane resistance in breast cancer cells *in vitro*, as its elevated expression correlates with low cytoplasmic concentration and decreased sensitivity to paclitaxel (33).

Breast tumor cell lines have been shown to develop P-gp mediated cross-resistance to drugs of the same class and in certain cases to drugs of different classes. Interestingly, breast

adenocarcinoma cells selected for resistance to doxorubicin showed several thousand-fold cross-resistance to both docetaxel and paclitaxel (34). An explanation for this may be that the anthracyclines first induce P-gp and that severe cross-resistance is observed because taxanes are a preferred substrate for P-gp compared to the anthracycline, doxorubicin (35). This observation may help to explain why patients were significantly less responsive to paclitaxel after late crossover from doxorubicin compared to treatment with doxorubicin after late crossover from paclitaxel (36). However, it appears unlikely that P-gp or other ABC drug efflux transporters play a prominent role in clinical resistance to taxanes in breast cancer patients.

2.5.2: Alterations in Microtubule Structure and Stability

Microtubules are dynamic polymers essential to the cell that can undergo elongation and shrinking with the ability to interact laterally with one another (29). They are required for a variety of cellular processes including transportation of macromolecules and organelles, maintaining and changing structure of the cytoskeleton, and mitosis and cell division (37). Microtubules are made up of α and β -tubulin subunits, which can be in either a polymerized or dimer form (29). It is widely accepted that taxanes bind to β -tubulin in the polymerized form and increase polymer stability (29). In the case of cell division, the increased stability of β -tubulin leads to cell cycle arrest in mitosis (29) and eventually to cell death. The β -tubulins are comprised of a variety of isotypes which vary at their C-termini (38). Molecular diversity among isotypes is accomplished by both the expression of distinct β -tubulin genes (38) and also post-translational modifications to β -tubulin gene products (39). Expression of certain β -tubulin isotypes is tissue-specific, while other isotypes are constitutively expressed (29). The functional specificity of different tubulin isotypes among tissues has yet to be determined (29).

Regardless of the β -tubulin isotype, polymers can form and the binding site for paclitaxel is only present in the case of the polymerized form of β -tubulin. Hence, it is suggested that selection for cancer cells in which the equilibrium between dimer and polymer has shifted towards the dimer form, could offer a survival advantage for a tumor that is treated with a microtubule-stabilizing agent, such as paclitaxel (29).

Microtubule dynamics are also controlled by the differential expression of tubulin isotypes, mutations within the tubulin genes, and also interactions with tubulin regulatory proteins. Tubulin regulatory proteins such as the microtubule-associated proteins (MAPs) or stathmin interact with tubulin to promote polymerization or disassembly, respectively (29). Increased stathmin mRNA levels have been measured in breast carcinoma tissue from patients with more aggressive disease (40). It is also possible that post-translational modifications to tubulin such as phosphorylation, polyglutamylation, polyglycylation among others, may alter the binding of tubulin regulatory proteins, microtubule dynamics, and thus taxane efficacy (29).

2.5.2.1: Differential Expression of Specific β -tubulin Isotypes

As mentioned, the tubulin isotype expressed in cells has an effect on the properties of polymer assembly and thus affects interactions with taxanes and microtubule dynamics. For example, microtubules assembled from β III-tubulin are considerably less sensitive to the suppressive effects of paclitaxel on their dynamics, than microtubules assembled from β II-tubulin (41). This suggests that selective expression of certain β -tubulin isotypes may affect the cellular sensitivity to taxanes.

A number of *in vitro* studies suggest a relationship between β III-tubulin isotype levels and taxane resistance. For example, one study examined tubulin isotypes and mutations in

paclitaxel-resistant cells by combined isoelectric focusing and mass spectrometry, and found that class III β -tubulin expression did, in fact, correlate with resistance to paclitaxel (42). Moreover, an association between class III β -tubulin expression and resistance to paclitaxel has been observed in a variety of human cancer cell lines of lung, ovarian, prostate and breast origin (43). However, another study showed that β I, β II, and β III-tubulin levels were decreased and β IV-tubulin levels increased when MDA-MB-231 cells were selected for taxane resistance (44). Nevertheless, there is evidence of β -III tubulin's role in tumor resistance to taxanes in cancer patients (see 2.4.1), suggesting that differential expression of β -tubulin isotypes may be an important mechanism of taxane resistance in breast tumors.

2.5.2.2: Point Mutations in Tubulin

The binding of taxanes to β -tubulin subunits in microtubules can also be affected by mutations in genes coding for either β - or α -tubulin (45). These mutations can affect the sensitivity of cells to taxanes by causing a change in microtubule dynamics. It has been observed that cells with specific β I-tubulin mutations become resistant to paclitaxel *in vitro* (46), and that some paclitaxel-resistant cell lines depend on paclitaxel for survival (29). A potential explanation for this is that certain tubulin mutations shift the equilibrium in favor of the dimer form, such that cells harboring these mutations become hypersensitive to drugs that bind the dimer form of tubulin such as colchicine and vinblastine (47). In some cases, the equilibrium is shifted to such an extent that the resulting lack of polymer stability compromises the cell's basic functions and thus paclitaxel's polymer stabilizing effects shift the polymer-dimer equilibrium in a more favorable direction, promoting survival (29).

Another form of taxane resistance can occur from a mutation in either α - or β -tubulin that alters the drug-binding site on β -tubulin polymers, such that it has less affinity for taxanes. *In vitro* reports of point mutations associated with taxane resistance in breast cancer cells have been reported, but in other studies, including clinical studies, no association between point mutations in tubulin genes and taxane resistance has been observed. For example, no mutations in β -tubulin genes were found when β -tubulin sequence information was compared between two docetaxel-resistant variants of the MDA-MB-231 and MCF-7 human breast adenocarcinoma cell lines and their drug-sensitive parental cell lines (44). A clinical study in 2003 also revealed that mutations in the class I β -tubulin gene did not predict response to paclitaxel in breast cancer patients (48). Thus, despite *in vitro* reports showing an association between β -tubulin mutations and taxane resistance, this association is not observed in breast cancer patients treated with taxanes.

2.5.3: Inhibition of Apoptosis

The arrest in mitosis caused by taxane-binding to microtubules appears to promote the induction of apoptosis. The trigger for apoptosis is governed by the effects of taxanes on key apoptotic regulatory proteins. For example, it is believed that taxanes induce hyperphosphorylation of BCL-2 and BCL-XL, which subsequently blocks their ability to bind to and antagonize the apoptosis-inducers BAX and BAK (49, 51). BAX and BAK are then free to dimerize and cause pore formation within the mitochondrial membrane, thus mediating apoptosis by the intrinsic apoptotic pathway (49–51). Taxanes also can cause BAX upregulation to promote apoptosis (49–51). It has also been suggested that paclitaxel can directly bind and sequester BCL-2, a microtubule-independent mechanism of cell death (52).

The function of BCL-2 is often regulated post-translationally by a variety of growth factor and cytokine signaling pathways (53). These pathways can drive BCL-2 upregulation and induce paclitaxel resistance (54). For example, exposure to estrogen in estrogen-responsive breast adenocarcinoma cells (MCF-7) is associated with an increase in BCL-2 levels and resistance to paclitaxel-induced apoptosis (55). Interestingly, one study found that induced recombinant ER α expression in ER-negative breast cancer cells caused resistance to paclitaxel by inhibiting apoptosis, while blocking ER α receptor activity in ER-positive breast cancer cells caused sensitization to paclitaxel (56). There is also clinical evidence that patients with ER-positive breast tumors are less responsive to paclitaxel than patients with ER-negative tumors (57–59).

Breast cancer cells selected for resistance to escalating doses of docetaxel were shown to have alterations in TNF signaling pathways. Specifically, the TNFR1 receptor, which promotes cellular apoptosis, became downregulated upon resistance to docetaxel (30). This downregulation of TNFR1 lead to increased activation of the transcription factor NF- κ B, which promotes expression of antiapoptotic survival genes such as c-FLIP (60) XIAP, and BCL-XL, which are known to cause chemotherapy resistance (61, 62).

2.5.4: Activation of Survival Pathways

A cell's tendency to live or die is determined by the net balance of opposing death and survival pathways. Induction of survival pathways in breast cancer cells is often associated with resistance to taxanes. Taxane-resistant breast adenocarcinoma cells have been observed to possess an amplified positive-feedback loop involving the TNF α -dependent activation of NF- κ B, which promotes expression of pro-survival genes. This involves the expression and secretion of

cytokines, which complete the loop by way of autocrine or paracrine signaling (30). Increased nuclear staining of NF- κ B in tumors (indicative of activated NF- κ B) has been shown to be associated with resistance to chemotherapy treatment with anthracycline- or taxane-containing regimens in breast cancer patients (63). Nevertheless, the true clinical relevance of such pathways in taxane resistance in breast cancer can only be determined through repeated clinical investigation.

2.6: Mechanisms of Resistance to Anthracyclines and Taxanes *In Vivo*

While providing significant insight into potential mechanisms of taxane or anthracycline resistance, the majority of the above *in vitro* studies fail to address important characteristics of human tumors that can impact on drug response and resistance. Such characteristics include their three-dimensional nature, the vasculature that provides nutrients and oxygen, and a complex tumor microenvironment comprised of surrounding stromal tissue, the extracellular matrix, and cells recruited by tumors (endothelial cells, fibroblasts, inflammatory cells of the immune system and pericytes). It is likely that some of these characteristics can account for the lack of relevance of some *in vitro* drug resistance mechanisms in clinical studies. This tumor microenvironment creates the potential for cells within a tumor to be deprived of oxygen and nutrients, evade drug exposure, and exhibit a reduced proliferation rate, all of which could present a barrier to taxane or anthracycline cytotoxicity.

2.6.1: Changes in Tubulin Isoform Expression

As mentioned previously, *in vitro* studies have shown that there may be a correlation between expression levels of specific tubulin isoforms and taxane resistance in breast cancer cells (43, 64). Clinical studies appear to support such a view, as one study showed that breast cancer patients with high levels of class I and class III β -tubulin transcripts are less likely to respond to docetaxel than patients with the following levels of tubulin transcripts: class I-low/class III-low, class I-high/ class III low or class I-low/class III high (65). Also supporting this study, high tumor levels of tubulin β -I and β -III transcripts were found to correlate with clinical resistance to paclitaxel in advanced breast cancer (66). While these reports are compelling, further studies are required to assess whether tumor levels of tubulin β -I and β -III transcripts can serve as an effective biomarker of taxane resistance in multiple cohorts of breast cancer patients.

2.6.2: Interactions with Stromal Cells

Interactions between epithelial and stromal tissue play an important role in the function of healthy mammary glands (67) and mediate suppression of transformation to preneoplastic phenotypes (68). It has been suggested that cancer could be a physiological response to an abnormal stromal environment in some cases (69), as reviewed by Barcellos-Hoff and Medina (70). In addition, stromal tissue can affect chemotherapy response through its tumor-supporting behavior (71).

Human cells communicate by secreting cytokines, chemokines, and growth factors that convey signals to nearby cells or travel through the bloodstream and affect more distal tissues. Activation of the innate immune response originates from the site of infection or inflammation, whereby signals are made available to components of the immune system, including monocytes, via the bloodstream. In breast cancer, signals originating from tumor or nearby stromal cells can

strongly affect the host (patient) and may affect tumor response to chemotherapy. Accumulation of tumor-associated macrophages has been associated with poor prognosis in breast carcinoma, as they are suggested to exhibit a tumor-supporting phenotype in some cases, which can include secretion of cytokines that promote proliferation, angiogenesis, and metastasis (71).

It has recently been demonstrated that stromal gene expression can be an important factor in the clinical outcome of breast cancer patients treated with adjuvant chemotherapy (72). In this study, tumor stromal samples were classified as being from a patient with good, poor or bad outcome after assessment of clinical status post-treatment. Stromal overexpression of a specific set of immune-related genes, including T cell and natural killer cell markers, typical of a T_{H1} type immune response, was correlated with a good clinical outcome in patients (72). On the other hand, stroma from individuals in the poor-outcome group showed markers of hypoxia and angiogenesis, along with a decrease in chemokines that stimulate natural killer cell migration and mediate pro-survival signals in T-lymphocytes (72, 73). In another clinical study, mesenchymal/stromal gene expression signatures were shown to be useful in predicting resistance to neoadjuvant chemotherapy in breast cancer (74).

2.6.3: Nutrient Deprivation, Hypoxia, and Acidity

Tumors are generally less vascularized than healthy tissue. As cells within a tumor reside farther from blood vessels, the level of nutrients falls and tumor cells in these areas tend to have decreased proliferation rates (75). It is suggested that since most anticancer drugs including taxanes and anthracyclines tend to be most toxic to rapidly dividing cells, slowly proliferating cells tend to be more drug-resistant (76). As nutrient levels are lower at distances further from vessels, so are pH and levels of molecular oxygen (77). Such hypoxic regions typically have

increased expression of P-gp (78), which as mentioned, can cause taxane or anthracycline efflux from tumor cells. It has been suggested that anthracyclines may rely on superoxide formation as a means of cytotoxicity (79) and thus tumor cells in hypoxic regions may be less likely to suffer an attack of this nature (77).

Low pH in the tumor microenvironment is typical, as cancers often rely more heavily on glycolysis than normal tissues (80, 81) and slower clearance of breakdown products (82–84). This can influence the cytotoxicity of anticancer drugs like doxorubicin, which are weakly basic. Protonation of such weak bases in acid environments could then result in decrease cellular drug uptake (84, 85).

2.6.4: Drug Penetration in Tumors

Both taxanes and anthracyclines are administered intravenously and must cross capillary vessel walls to reach cancer cells. For cells in the interior of tumors, this requires extensive diffusion through multiple layers of tumor cells (referred to as “packing density”) (76). By visualizing the location of doxorubicin through its natural fluorescence, it has been shown that high concentrations of doxorubicin are found within and around blood vessels, but concentrations of doxorubicin are considerably lower as the distance from the nearest blood vessel increases (86). It is suggested that the inability of both doxorubicin and epirubicin to penetrate deep into tumors may be the result of its sequestration in perinuclear endosomes and other organelles at the tumor surface or nearby host tissue (87).

2.6.5: Role of Tumor Initiating Cells in Anthracycline and Taxane Resistance

Solid tumors are generally heterogeneous, a product of their relatively high genetic instability. This results in tumors containing cells with a diversity of phenotypes, including rare cells exhibiting stem cell characteristics (quiescence, pluripotency, increased capacity for DNA repair) and both ABC transporter expression or dependence on surrounding stromal cells for survival (32). Such “stem cells” within tumors are referred to as tumor initiating cells (TICs)—due to their ability to initiate tumor formation when injected into mice. Such cells may have significant relevance in taxane and anthracycline resistance in patients with breast cancer.

TICs have been identified in a variety of cancers including multiple myelomas (88), leukemia (89, 90), colorectal (91), prostate (92), and hepatocellular carcinomas (93). Breast cancer TICs are defined by specific cell surface markers ($CD44^{+}/CD24^{-}/ALDH1^{+}$). Additionally, in many cases, breast cancer TICs have been shown to be dependent on developmental signaling pathways (94), particularly the Notch, WNT and Hedgehog pathways (94). Since TICs tend to possess properties similar to less differentiated cells, they may possess the ability to adapt to the adverse conditions caused by chemotherapy treatment (94, 95). In addition, since TICs are relatively quiescent, they are less sensitive to chemotherapy agents that target rapidly dividing cells, such as the taxanes or anthracyclines. They also may overexpress ABC drug transporters, which are known to play a role in resistance to both taxanes and anthracyclines, as mentioned in previous sections (94, 95). Nevertheless, there has been controversy about the cell surface markers that define breast TICs and which stem cell markers are correlated with chemotherapy resistance (95, 96). “Basal-like” breast cancers are associated with poor patient prognosis and have many of the properties of TICs (97), but such cancers remain some of the most chemotherapy responsive tumors (98). Moreover, while clear subtypes of breast cancer have been identified through gene profiling studies (99), and while these subtypes differ in response to

adjuvant chemotherapy (100), there are currently no pre-treatment genetic or protein biomarkers that can definitively distinguish between tumors that are responsive to anthracycline or taxane-chemotherapy regimens and those that are not (101).

2.7: Management of Breast Cancer Patients with Drug-Resistant Tumors

Even if the appropriate biomarkers can be found to identify chemotherapy resistant tumors, the challenge of how to manage patients with such tumors remains. Typically upon failure to respond to chemotherapy with anthracyclines and taxanes, treatment moves to other chemotherapy drugs in the adjuvant setting or to surgery and/or radiation therapy in the neoadjuvant setting. Strategies used to treat drug-resistant breast cancer involve the employment of drugs with mechanisms of action distinct from taxanes and anthracyclines, including capecitabine (102), navelbine, gemcitabine (103), and carboplatin.

Currently there has been little success in restoring drug sensitivity to patients whose tumors have acquired resistance to anthracyclines and taxanes (3). With increased knowledge of clinically relevant drug resistance mechanisms, it may become possible to interfere with these mechanisms to restore chemotherapy sensitivity. An early example of attempts to re-establish drug sensitivity by interfering with a drug-resistance mechanism involves the employment of P-gp inhibitors in patients with chemotherapy-resistant tumors (104). Two such inhibitors, Verapamil and Tariquidar, were found to restore sensitivity to doxorubicin in drug-resistant cells *in vitro* (104), but had little effect on restoring clinical response to anthracycline- or taxane-containing chemotherapy regimens (105).

Another possible mechanism to restore sensitivity to chemotherapy regimens in breast cancer patients may involve the use of chloroquine. Chloroquine (Resochin) was originally developed as a drug to prevent malarial infections in humans (106). Its use has been expanded to include treatment for autoimmune disorders such as rheumatoid arthritis and recently as a radio-sensitizing or chemo-sensitizing agent in cancer and HIV chemotherapy (107–110). In the case of cancer treatment, chloroquine is thought to act by inhibiting autophagic survival while activating apoptotic pathways (109, 110). This occurs because chloroquine preferentially accumulates in lysosomes of the cells where the pH of the lysosomes traps the chloroquine (107). Additionally, chloroquine permeabilizes the lysosomes allowing for the release of lysosomal enzymes into the cytosol (107). Thus, chloroquine may sensitize tumor cells to radiotherapy or chemotherapy by interfering with autophagic survival pathways induced upon exposure to chemotherapy agents (107). Several clinical trials are currently under way to assess the efficacy of chloroquine as a possible tool to restore sensitivity to chemotherapy agents, such as the anthracyclines and taxanes. Research is also being performed on other autophagy inhibitors as sensitizing agents for chemo-resistant tumors.

Given that patient tumors vary in response to chemotherapy agents (both prior to and after previous rounds of chemotherapy), an additional approach to manage breast cancer patients would be to accurately assess tumor response to chemotherapy early in treatment, such that patients with non-responding tumors could be quickly switched to other downstream regimens such as surgery, radiation therapy, or other chemotherapy drugs. A recent study revealed that locally advanced breast cancer patients exhibiting a pathologic complete response to epirubicin/docetaxel chemotherapy post-treatment exhibited significant reductions in RNA integrity during chemotherapy (111). This “response biomarker” may be of particular value in

patient management, if tumor response can be determined after one or two cycles of chemotherapy. The true value of this biomarker will only be determined through additional studies involving the assessment of multiple cohorts of breast cancer patients at various cycles during chemotherapy treatment.

2.8: Concluding Remarks

Anthracyclines and taxanes are powerful chemotherapy drugs used in the treatment of breast cancer, in particular for those patients that achieve a pathologic complete response to treatment with these agents. However, the majority of patients exhibit innate or acquired resistance to anthracycline- or taxane-containing regimens. While much has been learned from *in vitro* and *in vivo* studies on resistance to anthracyclines and taxanes in breast tumor cells, it appears likely that breast tumors evade the action of these agents through multiple mechanisms. Moreover, these mechanisms likely vary among patients and among the cell population within a given tumor. This makes it difficult to predict chemotherapy response and to identify a single small molecule that will block innate or acquired drug resistance. Nevertheless, significant advancements have been made in understanding the molecular diversity of breast cancers and their differential sensitivity to anthracyclines and taxanes. These tools are helping guide the oncologist in assessing a particular patient's risk of treatment failure. In addition to such predictive biomarkers, the development of response biomarkers may help confirm drug resistance early in treatment, such that non-responding patients can be moved more rapidly to alternate and potentially more beneficial treatments. The development of agents to prevent or combat resistance to anthracyclines and taxanes in select or multiple cohorts of breast cancer patients would help further improve the therapeutic benefit to patients with breast cancer. Given

that the majority of patients do not receive a survival benefit from adjuvant or neoadjuvant chemotherapy with anthracycline and taxanes (112, 113), there is still significant and challenging work to be done.

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Chapter 3

Role of Drug Metabolism in the Cytotoxicity and Clinical Efficacy of Anthracyclines

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3.1: Author Contributions

RN authored the introduction section A as well as generated **Figure 3-1** and **Table 3-1**. SC authored the section pertaining to 2 electron reduction of anthracyclines, section B. DE authored the section pertaining to one electron reduction of anthracyclines, section C. JPJM authored the section pertaining to reductive deglycosylation of anthracyclines, section D. KMB authored the section pertaining to strategies of improving clinical response, section E. KMB also generated **Figure 3-2** with intellectual input from all authors. AMP authored the abstract and concluding remarks as well as participated in the conception, outline and drafting of the manuscript. All authors read and proved the manuscript.

3.2: Abstract

Many clinical studies involving anti-tumor agents neglect to consider how these agents are metabolized within the host and whether the creation of specific metabolites alters drug therapeutic properties or toxic side effects. However, this is not the case for the anthracycline class of chemotherapy drugs. This review describes the various enzymes involved in the one electron (semi-quinone) or two electron (hydroxylation) reduction of anthracyclines, or in their reductive deglycosidation into deoxyaglycones. The effects of these reductions on drug antitumor efficacy and toxic side effects are also discussed. Current evidence suggests that the one electron reduction of anthracyclines augments both their tumor toxicity and their toxicity towards the host, in particular their cardiotoxicity. In contrast, the two electron reduction (hydroxylation) of anthracyclines strongly reduces their ability to kill tumor cells, while augmenting cardiotoxicity through their accumulation within cardiomyocytes and their direct effects on excitation/contraction coupling within the myocytes. The reductive deglycosidation of anthracyclines appears to inactivate the drug and only occurs under rare, anaerobic conditions. This knowledge has resulted in the identification of important new approaches to improve the therapeutic index of anthracyclines, in particular by inhibiting their cardiotoxicity. The true utility of these approaches in the management of cancer patients undergoing anthracycline-based chemotherapy remains unclear, although one such agent (the iron chelator dexrazoxane) has recently been approved for clinical use.

Keywords: Anthracyclines, anti-tumor effects, cardiotoxicity, deoxyaglycone, hydroxylation, metabolites, optimization, semi-quinone, therapeutic index.

3.3: Introduction

Since their introduction in the 1960's, anthracyclines have been used in the treatment of many neoplastic diseases, including both solid tumors and hematological cancers. They are planar molecules consisting of a rigid hydrophobic tetracycline ring, with a daunosamine sugar attached through a glycosidic bond (1). The quinone and hydroquinone substitutions on two of four planar rings are important in their metabolism (1). Used as a single agent or as part of a regimen, the anthracyclines are key components of neoadjuvant, adjuvant, curative, or palliative treatments for several types of malignancies. Derived from the pigment-producing *Streptomyces peucetius* bacterium, doxorubicin (DOX) and daunorubicin (DNR) are two naturally occurring anthracyclines (2). Due to their success in treating cancers from various tissue types, a significant amount of effort has been devoted into creating and characterizing novel anthracyclines. This has resulted in the development of approximately 2,000 anthracycline analogs. A number of these analogs are now in widespread clinical use, including idarubicin, epirubicin, carminomycin, pirarubicin, aclarubicin, valrubicin and zorubicin (3, 4). As with other chemotherapy agents, the clinical success of anthracyclines is compromised by innate or acquired resistance to these agents (5) and by their significant toxic side effects in cancer patients, in particular cardiotoxicity (6, 7). Consequently, all anthracyclines have an associated maximum recommended cumulative dose in an effort to avoid congestive heart failure.

DNR and DOX, the first anthracycline antibiotics to be isolated over the past 50 years, are among the most effective antineoplastic agents currently used in the treatment of human cancers. DNR is used mainly to treat acute lymphoblastic or myeloblastic leukemias, while DOX has efficacy against both solid and non-solid tumors. The latter is widely used for the treatment of breast cancer, Wilms' tumors, soft tissue sarcomas, leukemias, Hodgkin's disease, non-

Hodgkin's lymphomas and several other cancers (8). Although differing from DOX by a single hydroxyl group, this alteration in structure gives DNR distinct reaction kinetics (9). Nevertheless, the use of both DOX and DNR is limited by their toxic side-effects within the host, including necrosis of tissue at the injection site, mucositis, alopecia, nausea, vomiting, stomatitis, and cumulative cardiotoxicity. Consequently, the maximum recommended cumulative doses for DNR and DOX are set at 550 mg/m² and 450-550 mg/m², respectively (10, 11).

Epirubicin (EPI) is obtained by an axial-to-equatorial epimerization of the 4'-hydroxyl group of DOX (**Figure 3-1**). It is currently widely used to treat carcinomas of the breast, stomach, gut, endometrium, lung, ovary, esophagus, and prostate (as well as soft tissue sarcomas) (12). While EPI has almost equivalent antitumor activity to that of DOX, it possesses different pharmacokinetic and metabolic characteristics. For example, EPI is more glucuronidated, which facilitates excretion in bile and urine. It therefore has a greater margin of safety and has almost double the recommended cumulative dosing of DOX (900-1,000 mg/m² for EPI) (13).

Idarubicin (IDA), an analog of DNR, lacks the C-4 methoxy group and has been shown to have improved activity for the treatment of acute myelogenous leukaemia. It is also found to be active against multiple myeloma, non-Hodgkin's lymphoma, and breast cancer (12). IDA is the only anthracycline that can be administered orally or through intravenous injection. The absence of a methoxy group in IDA's structure (**Figure 3-1**) results in a longer half-life than DNR and significantly enhances lipophilicity. This results in more rapid cellular uptake, superior DNA-binding capacity, and consequently greater cytotoxicity compared to DOX and DNR (14).

Comparative information regarding the half-life and toxicities of the above anthracyclines is presented in **Table 3-1**.

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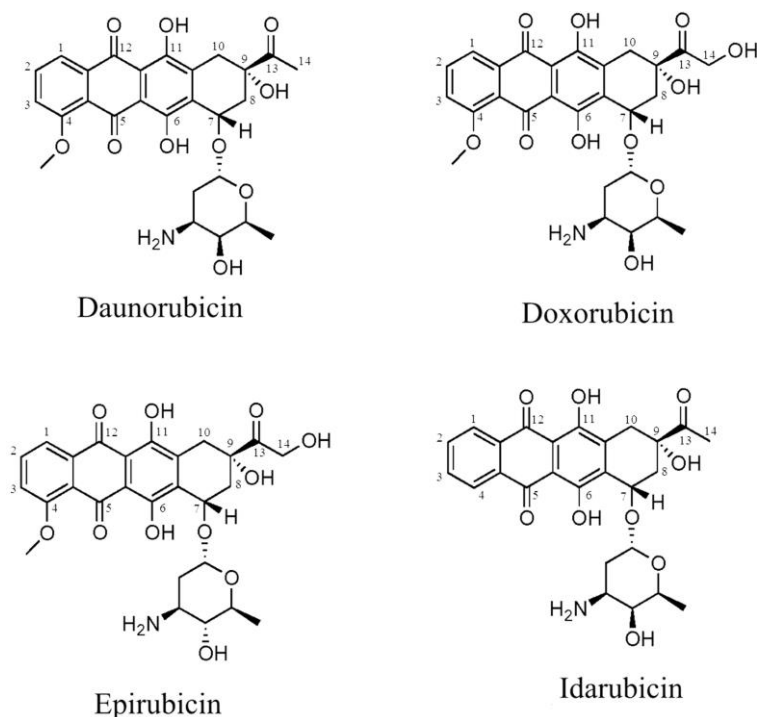


Figure 3-1 Chemical structures of Daunorubicin (DNR), Doxorubicin (DOX), Epirubicin (EPI) and Idarubicin (IDA).

The mechanism by which anthracyclines enter cells is still not completely known, but one mechanism that has been demonstrated involves their passive diffusion through the plasma membrane, followed by their selective transport into the nucleus by binding to proteasomes (15). Once in the nucleus, the anthracyclines dissociate from proteasomes and bind to DNA due to their higher affinity DNA (16, 17). Anthracyclines are thought to inhibit the proliferation of rapidly dividing cells through multiple mechanisms, including their ability to intercalate between and cross-link DNA strands, to alkylate DNA, and to inhibit topoisomerase II (18-20). These

actions are also highly effective in preventing DNA unwinding and strand separation, thereby blocking DNA replication and transcription (19). In addition, the ability of anthracyclines to generate highly reactive free radicals can result in abundant damage to DNA and to the plasma membrane through lipid oxidation (21-24). In addition to their cytostatic and cytotoxic effects against tumor cells, anthracyclines have been documented to accumulate in other tissues such as the liver, heart, white blood cells, and bone marrow contributing to their systemic toxic side effects (25).

In humans, it is estimated that approximately 50% of DOX is eliminated from the body without any change in its structure, while the remainder of the drug is processed through three major metabolic pathways (25). Metabolism of anthracyclines occurs through hydroxylation, semiquinone formation, or deoxyglycone formation, which can result in the formation of metabolites that either augment or suppress the anticancer properties of anthracyclines (26, 27).

Hydroxylation of anthracyclines at the C-13 carbonyl group, more commonly referred to as two electron reduction, results in the formation of secondary alcohol metabolites that have been implicated in anthracycline-induced cardiotoxicity (14). This major pathway of anthracycline biotransformation is mediated by a heterogeneous family of cytosolic NADPH-dependent carbonyl (CBR) and aldo-keto (AKR) reductases (collectively referred to as carbonyl reducing enzymes) that catalyze the formation of daunorubicinol (DNROL), doxorubicinol (DOXOL), epirubicinol (EPIOL), or idarubicinol (IDAOL) from their parent drugs (28). The AKRs are the primary enzymes involved in DOX hydroxylation in the human heart, whereas the CBRs play more of a role in DNR hydroxylation (29). The hydroxylation reactions occur in all cell types as the enzymes involved are ubiquitous, and have also been studied extensively in red blood cells, liver, and kidney (30).

The one electron reduction of anthracyclines is catalyzed by cytochrome P-450 reductase (CPR), NADH dehydrogenase (NDUFS), nitric oxide synthase (NOS), and xanthine oxidase, leading to the conversion of the quinone moiety of anthracycline drugs to a semiquinone radical (17). Although this radical is stable under anoxic conditions, in the presence of oxygen, the semiquinone radical is readily re-oxidized to regenerate the parent quinone and results in the

Drugs	Maximum Recommended Dose	Half-Life (hr)	Side Effects
Daunorubicin	550 mg/m ²	14-20	Cardiotoxicity, mucositis, myelosuppression, nausea, vomiting, alopecia
Doxorubicin	450 - 600 mg/m ²	1-3	Cardiotoxicity, mucositis, nausea, vomiting
Epirubicin	900-1,000 mg/m ²	33	Myelosuppression, cardiotoxicity, nausea, vomiting, mucositis
Idarubicin	>160 mg/m ²	12-27	Cardiotoxicity, myelosuppression mucositis, nausea, vomiting, alopecia

Table 3-1 Comparison of the four major anthracyclines used in clinical oncology and their differences in clinical use, pharmacokinetics, and toxicities.

generation of a superoxide anion and hydrogen peroxide, thereby increasing the formation of reactive oxygen species (ROS). The resulting free radicals can cause peroxidation of lipids within cellular membranes, protein aggregation, and cell death (31). This redox cycling of DOX, DNR, and other anthracycline analogues has been observed in the cytoplasm, mitochondria, and sarcoplasmic reticulum and has been implicated in the production of toxic aldehydes that are able to escape from the cell and contribute to anthracycline toxicity (8).

The final metabolic pathway, deglycosidation, accounts for approximately 1-2% of anthracycline metabolism (29). The reductive cleavage of the glycosidic bond and the side chain

carbonyl group results in the formation of 7-deoxyaglycones and hydroxyaglycones (32). It has been reported that this reaction is catalyzed by poorly characterized NADPH-dependent hydrolase- and reductase type glycosidases (NADPH quinone oxidoreductases (NQO1) and NADPH-cytochrome P450 reductase (CPR)) along with the involvement of xanthine dehydrogenase (XDH) (29). The formation of hydroxyaglycones results from NADPH-dependent enzymes that are present in the cytosol, whereas the 7-deoxyaglycone formation may be initiated by microsomal or mitochondrial oxidoreductases (32). Several studies report that the anthracycline aglycones may generate ROS, but the resulting 7-deoxyaglycone metabolites have been shown to possess no cytotoxic activity (33). The aglycones produced have a higher lipophilicity than the parent anthracyclines, and are thought to intercalate into mitochondrial membranes (34). Moreover, studies suggest that the anthracycline aglycones may cause myocardial damage due to their prominent oxidizing properties that divert more electrons to oxygen in the mitochondria (35). While anthracycline metabolic pathways and metabolites vary from tissue to tissue and between *in vitro* and *in vivo* conditions, the two electron reduction (hydroxylated) product is generally the predominant metabolite for anthracyclines, with considerably lower percentages of the aglycone, 7-deoxy aglycone and 7-deoxy hydroxylated aglycone products (33, 36).

Having summarized the various pathways by which anthracyclines are metabolized in humans, this review will describe how this metabolism can affect the above-described biochemical and biological properties of this important class of chemotherapy agents. We will focus particularly on how the metabolism of anthracyclines affects their ability to combat the growth of tumors and to produce toxic side effects in patients. In addition, we will explore how variations in anthracycline metabolism within cancer patients and their tumors can impact

treatment efficacy and discuss recent strategies to improve the therapeutic index of anthracyclines. A schematic summary of the major points of this review is provided in **Figure 3-2**.

3.4: Two Electron Reduction of Anthracyclines (Hydroxylation)

Anthracyclines hydroxylated at the C13 position are more polar than the parent compound and have higher water solubility. However, contrary to what might be expected, these hydroxylated metabolites are excreted from certain tissues at slower rates than the parental compound. They are also considerably less potent antineoplastic agents, while having greater cardiotoxicity due to their enhanced ability to accumulate in cardiac tissue relative to the parent compounds (37). Peters *et al.* showed that the levels of DOXOL in rats administered DOX were greatly enhanced in cardiac muscle compared to other tissues, including liver and skeletal muscle. The reason for this preferential accumulation of DOXOL in cardiac muscle is unknown (38). With the exception of IDA, the C-13 metabolites for DNR, DOX and EPI are all less cytotoxic than their unhydroxylated forms (39, 40). IDAOL is equally cytotoxic as IDA (39, 40).

3.4.1: Carbonyl Reductases (CBRs)

CBRs have recently been extensively reviewed regarding their function as drug metabolizing agents by Malatkova and Wsol in 2014 (41). Thus, for this review, we will only briefly discuss their role in anthracycline metabolism. While AKRs and CBRs are both carbonyl reducing enzymes, this section focuses specifically on the carbonyl reductases CBR1 and CBR3 (41, 42). These enzymes are found in a wide variety of tissues with a very broad range of substrates. They are generally associated with detoxifying toxic substrates to protect cells and organs (44, 45). CBR1 is the predominant reductase that hydroxylates DOX in the liver, kidney,

and gastrointestinal tract (43). In a recent study, Kassner and colleagues (43) assessed the relative roles of the AKRs and CBRs in the two electron reduction (hydroxylation) of DOX. Interestingly, the kidney, liver, and gastrointestinal tract (which are collectively responsible for the clearance of DOX from the body) express carbonyl reducing activity (ies) with an apparent K_m of 140 μ M. Enzymes with K_m values in this range include carbonyl reductase 1 (CBR1) and AKR1C3. CBR1 was found to be expressed in the above three organs at higher levels than AKR1C3, while the latter exhibited a higher catalytic efficiency. An inhibitor capable of discriminating between the CBR1 and AKR1C3 activities was able to equally block the carbonyl reducing activity of CBR1 and human liver cytosol, but not AKR1C3. This suggests that CBR1 plays the predominant role in the liver's ability to hydroxylate DOX. As previously discussed, this is distinct from the human heart, where AKRs play the predominant role in the hydroxylation of DOX (29).

3.4.2: Aldo-Keto Reductases (AKR)

All members of the AKR superfamily of proteins reduce ketones and aldehydes into secondary and primary alcohols in a NADPH-dependent manner (44). The superfamily contains more than 140 members, sub-categorized into 15 families. AKR families share a minimum of 40% protein sequence identity, while their sub-families share >60% sequence identity (44). Individual AKRs are named beginning with AKR, followed by: a number indicating the family of AKRs, a letter denoting the sub-family, and finally a number designating the individual member. The AKR1 family is the largest of the 15 families, and is one of three families including the mammalian AKRs (44). While the AKR superfamily is comprised of many members, this review focuses primarily on family members that are known to hydroxylate anthracyclines and/or whose expression changes upon selection for anthracycline resistance.

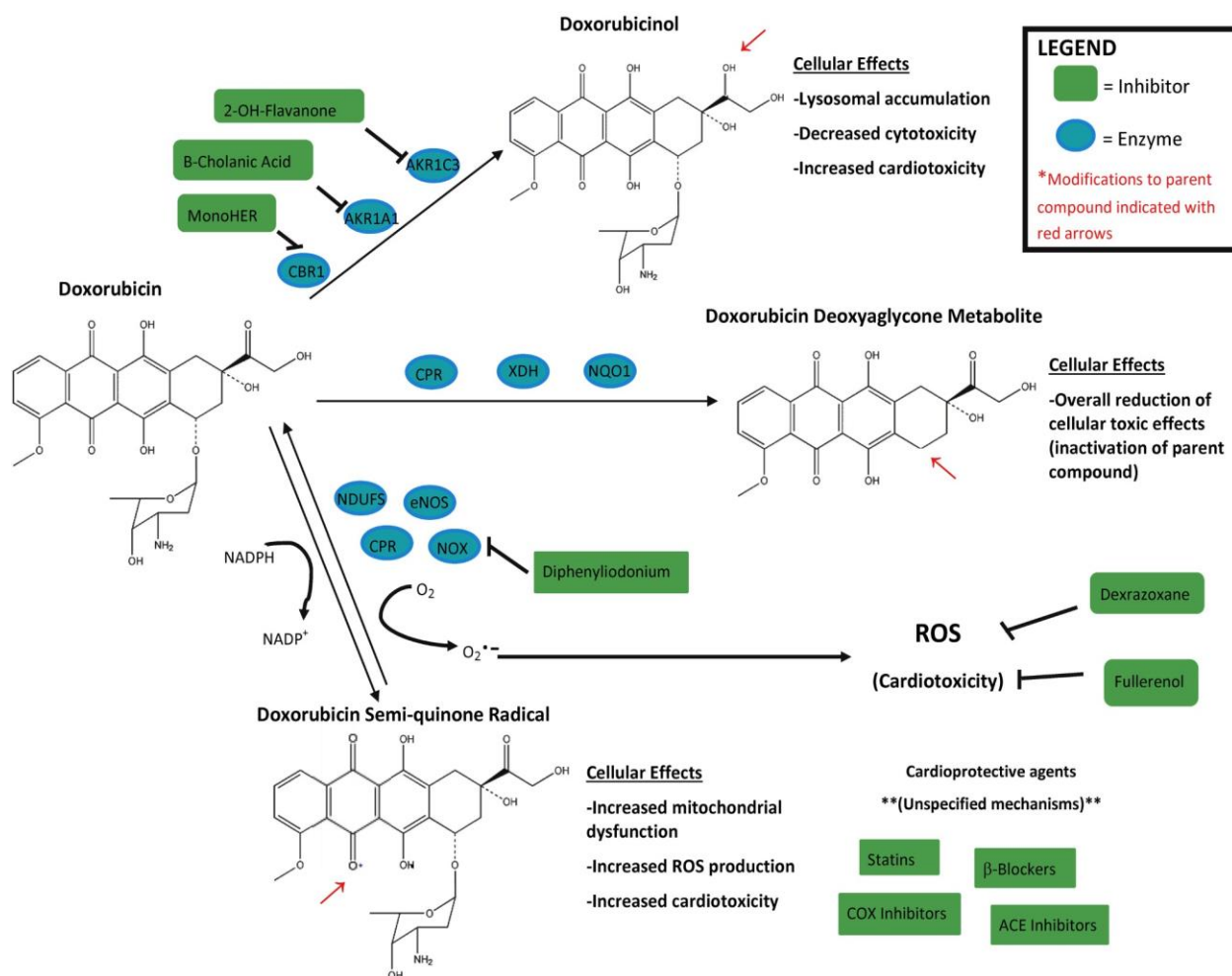


Figure 3-2 Main pathways of intracellular doxorubicin (DOX) biotransformation in mammalian cells, including catalytic enzymes involved in its metabolism and their inhibitors. The downstream effects of the metabolites are also listed. Similar pathways are involved in metabolism of other anthracyclines.

3.4.3: AKR1 Family

The AKR1 family contains six sub-families, the largest of which is the AKR1C subfamily. AKR1C members primarily function as steroid metabolizing enzymes, with AKR1C1 to 1C4 sharing >86% sequence homology (44, 45). AKR1C3, also known as 17-hydroxysteroid-dehydrogenase type V (17-HSD) or 3-hydroxysteroid-dehydrogenase type I (3-HSD),

plays a role in the conversion of androstenedione into testosterone and estrone (E1) into estradiol (E2) (45-47). In addition, AKR1A1, AKR1C1, AKR1C2, and AKR1C3 have been shown to play a role in the metabolism and detoxification of chemotherapy agents (48, 49), with AKR1A1 being specifically identified as the likely AKR involved in the conversion of the anthracycline DOX into DOXOL in cancer patients (50). A variety of AKR members (AKR1A1, AKR1B1, AKR1B10, AKR1C1, AKR1C4 and AKR7A2) also play a role in the detoxification of reactive aldehydes, some of which may be created by anthracycline-generated ROS (44, 45, 51). AKRs are differentially expressed in various tissues throughout the body. AKR1A1 is most highly expressed in kidneys, followed by the liver with the lowest expression being in the lungs (44). AKR1B1, AKR1B10 and the AKR1C isoforms 1 through 4 have been shown to be primarily expressed in the liver, with AKR1C4 being exclusively expressed in this organ. While most studies focus on the AKR1 family of enzymes and their ability to metabolize anthracyclines, it should be noted that AKR7A2 has also been shown to have some metabolic activity towards these substances in a wide variety of organs (52).

While belonging to a different family of enzymes, this ability of AKR7A2 demonstrates the potential that other AKR family members may play yet undiscovered roles in anthracycline metabolism. Due to their roles in xenobiotic detoxification and steroid synthesis, AKR1C isoforms 1 through 3 are also highly expressed in the intestine, mammary glands, prostate, ovary, and lungs (44, 53), with AKR1C3 the predominant AKR in mammary glands. The widespread distribution of AKRs in tissue ensures that a substantial amount of the anthracyclines administered to cancer patients is rapidly hydroxylated.

3.4.4: Specific Roles of AKR1C2, AKR1C3 and AKR1B10 in Anthracycline Metabolism and Resistance

While several AKRs have been studied for their role in anthracycline metabolism, only a subset specifically affect the biochemical and cytotoxic properties of anthracyclines. Heibein *et al.* showed recently that while exogenously added DOX localizes to the nucleus of breast tumor cells, exogenous DOXOL accumulates in lysosomes (54). This suggests that the hydroxylation of this anthracycline prevents the drug from entering the nucleus, possibly due to its poorer affinity for binding to proteasomes. In addition, Heibein *et al.* observed that DOXOL (54) had significantly less affinity for DNA compared to DOX, which may also account for the former's lack of localization to the nucleus. We also showed that selection of MCF-7 breast tumor cells for DOX resistance resulted in increased transcription of the *AKR1C2*, *AKR1C3* and *AKR1B10* genes (54, 55). Interestingly, the AKR inhibitor β -cholanolic acid was able to restore localization of DOX (but not DOXOL) to the nucleus in DOX-resistant cells (54), suggesting that the inhibitor blocked DOX hydroxylation. Consistent with this view, the intracellular DOX concentration in the DOX resistant cells increased in the presence of β -cholanolic acid (54).

As stated previously, anthracyclines have been shown to be potent inhibitors of DNA topoisomerase II (20) and to induce substantial DNA damage (23) in tumor cells. Interestingly, the hydroxylation of the C-13 carbonyl group in two anthracyclines (DOX and IDA) was found to have little effect on their ability to inhibit topoisomerase II (40). Moreover, both of these anthracyclines and their alcohol metabolites had a strong ability to induce DNA damage. In fact, in isolated nuclei, the hydroxylated forms of the drugs induced twice the DNA damage as their parental compounds (40). Given that hydroxylation of most anthracyclines dramatically reduces

their cytotoxic effects (39, 40), the above findings suggest that their cytotoxicity (at least *in vitro*) is not dependent upon their ability to inhibit topoisomerase or to induce DNA damage.

3.4.5: Host toxicity

The major toxic side effect of anthracyclines in cancer patients relates to the high cardio toxic properties of their hydroxylated metabolites, particular for DOX. DOXOL appears to have a direct effect on excitation/contraction coupling in ventricular myocytes (56). In humans, the hydroxylated anthracyclines induce delayed onset cardiomyopathy, which can manifest itself during the course of treatment or even weeks or months post-treatment (32). In recent years, studies on the host toxicity of anthracyclines have been largely focussed on the role that carbonyl reductases play in this phenomenon. In one study, mice exhibiting a *CBR1* null allele were shown to exhibit substantially reduced cardiotoxicity compared to wildtype mice, when both were administered DOX (57). In another study, the overexpression of human carbonyl reductase in transgenic mice advanced the development of DOX-induced cardiotoxicity (58). Mechanistically, it is generally believed that the anthracyclines become metabolized within cardiomyocytes by carbonyl reducing enzymes into their alcohol metabolites. These metabolites are not easily expelled from the myocytes, where they increasingly activate calcium release from calcium release channels (59). This leads to a general dysfunction in the cardiomyocyte which leads to cell death and eventual cardiac myopathy. Interestingly, a subsequent study by Shadle *et al.* (60) demonstrated that the anthracycline DNR opened calcium release channels in sarcoplasmic reticulum preparations from rabbit atria with a potency 20 times that of the quinone-deficient analogue, 5-iminodaunorubicin. Moreover, neither anthracycline induced free-radical formation, suggesting that DNR impairs the contraction of the myocardium by

interference with sarcoplasmic reticulum function via a mechanism not involving free radical formation.

The specific involvement of polymorphisms in *CBR* genes and patient cardiotoxicity after anthracycline treatment remains controversial. In one study, two polymorphisms in two carbonyl reductase genes (*CBR1* 1096G>A; *CBR3* V244M) were identified as genetic biomarkers associated with susceptibility to cardiac damage from anthracycline treatment in pediatric oncology patients (61). In contrast, another study found no relationship between any polymorphisms in *AKR* or *CBR* genes and cardiotoxicity associated with anthracycline treatment (62). Despite this discrepancy as to the role of *CBR* polymorphisms in the cardiotoxicity of anthracyclines, the link between carbonyl reductases and cardiotoxicity resulting from anthracycline treatment is supported by a number of additional recent studies (63-65). A convincing line of evidence supporting this link is a very recent study showing that the compound, 23-hydroxybetulinic acid, inhibits carbonyl reductase activity and was able to reduce both the accumulation of DOXOL in mice hearts and DOX-induced cardiotoxicity (66). There is less support for the role of *AKR*s in promoting the cardiotoxicity of anthracyclines. For example, DOX is considered to have the greatest effect on cardiotoxicity; however, it has the lowest affinity for *AKR* enzymes (48), in particular for *AKR7A2*, which has been shown to have very low specific activities towards DOX and DNR (52). In contrast, IDA, which has one of the lowest cardiotoxicities of the anthracyclines has one of the highest affinities for metabolism by *AKR*'s (48).

3.5: One Electron Reduction of Anthracyclines (Semiquinone Formation)

Anthracycline semiquinone formation involves a one-electron reduction of the quinone moiety. This reaction is thought to be catalyzed by a variety of cellular NADH- and NADPH-

dependent reductases. The semiquinone metabolites of anthracyclines are generally agreed to be more cytotoxic than the parental molecules, both in combating tumor growth and in their effects on host tissues. The semiquinone anthracycline may elicit its cytotoxic effects by facilitating the alkylation of cellular macromolecules (67) or through the generation of oxygen radicals (68, 69). Enzymes that are likely to catalyze the one-electron reduction of the anthracycline include NADPH-cytochrome P450 reductase (70), endothelial nitric oxide synthase (71), and NADH dehydrogenase (ubiquinone) Fe-S (72).

3.5.1: Host toxicity via Anthracycline Semiquinone Metabolites

Along with their efficacy in treating a variety of human neoplasms, the anthracyclines are known to cause various hematological toxicities, in particular neutropenia associated with DOX treatment (73). Along with myelosuppression, DOX treatment can also cause symptoms such as nausea, vomiting, and cardiac arrhythmias, all of which are clinically manageable (74). It is currently unclear whether these effects are dependent on the reductive conversion of anthracyclines to their semiquinone form; however, cardiotoxicity is thought to be particularly dependent on the formation of the semiquinone metabolite (71, 72).

At the subcellular level, anthracycline-induced cardiomyopathy is thought to be associated with the drug's ability to induce mitochondrial dysfunction (68, 69). As with other muscle tissues, cardiomyocytes rely on mitochondria for ~90% of their ATP production (75). In fact, mitochondria make up about 20-40% of the cardiomyocyte volume, which is greater than that of skeletal muscle (76). The presence of particular reductive enzymes within heart mitochondria may account for the ability of anthracycline containing regimens to induce cardiotoxicity in cancer patients. Anthracyclines and their semiquinone metabolites have been

well studied for their often irreversible damage to the heart, which can become apparent between four and twenty years after the completion of chemotherapy (77).

3.5.1.1: NADH dehydrogenase (Ubiquinone) Fe-S (NDUFS)

NADH dehydrogenase (ubiquinone) Fe-S is associated with complex I of the respiratory chain within the inner mitochondrial membrane. It typically catalyzes the transfer of electrons from NADH to coenzyme Q10 (78). This high molecular weight iron sulfur protein complex (~1,000 kDa) consists of 45 subunits, seven of which are encoded by mitochondrial DNA and the remainder by the nuclear genome (79). Anthracycline semiquinone formation is particularly favorable in the membranes of heart mitochondria. A study by Nohl *et al.* found that mitochondria from the heart easily shuttle single electrons to DOX to promote semiquinone formation. In contrast, they found that liver mitochondria are ineffective in producing semiquinones from DOX (72). Nohl *et al.* further suggested that NADH dehydrogenase of complex I catalyzes DOX semiquinone formation in heart mitochondrial membranes and that this enzyme is absent in liver mitochondria. The semiquinone metabolite can be reoxidized non-enzymatically to produce superoxide radicals that can lead to DOX aglycone semiquinone formation. The latter metabolite, due to its increased lipophilicity, can accumulate in the inner mitochondrial membrane where it disrupts other electron carriers of the respiratory chain (72).

3.5.1.2: Endothelial Nitric Oxide Synthase (eNOS)

Endothelial nitric oxide synthase (eNOS) is a membrane-bound enzyme located in coronary endothelial cells (80). Typically, this enzyme oxidizes the amino acid L-arginine to produce L-citrulline and the nitric oxide radical NO[•]. This process involves the transfer of electrons from NADPH, or FAD, among other electron donors, as well as cofactors including

Ca^{2+} and the Ca^{2+} -binding protein calmodulin (81, 82). The depletion of one or more of these substrates or cofactors can impair NO^{\bullet} synthesis (83). eNOS accounts for most of the NO^{\bullet} production in the heart, and regulation of its activity controls the amounts of nitric oxide produced (80). In the cardiovascular system, the basal release of NO^{\bullet} is necessary for healthy vasodilatory tone (84). Production of this vasodilator regulates blood pressure and vascular flow to tissues, including the brain, heart and lungs (84). Supporting the role of eNOS in anthracycline semiquinone formation, Vasquez-vivar *et al.* demonstrated that DOX binding to the reductase domain of eNOS resulted in a one-electron reduction of DOX to DOX semiquinone in a NADPH dependent manner (71). They also showed that this metabolite was able to reduce oxygen independently of the enzyme, producing superoxide ($\text{O}_2^{\bullet-}$) (71).

Among its many effects on cells, DOX has the ability to induce eNOS gene transcription and increases the activity of the enzyme in bovine aortic endothelial cells (85). Moreover, an antisense RNA targeting eNOS gene transcripts was able to abrogate DOX-induced apoptosis (85), suggesting that semiquinone formation was associated with DOX cytotoxicity. Similarly eNOS appears to play a role in mediating the toxic side effects of DOX in the host, since Neilan *et al.* have shown that eNOS gene knock-out in mice protected against DOX-induced cardiac dysfunction, injury, and mortality (86). These investigators further demonstrated that overexpression of eNOS transcripts in cardiomyocytes of mice resulted in greater increases in left ventricular dimensions and larger reductions in systolic function after a single dose of DOX than in eNOS knockout or wild-type mice. DOX administration led to superoxide production in the hearts of wild-type mice but not in eNOS-deficient mice and DOX-induced superoxide production was even greater in eNOS-overexpressing mice than in wild-type mice (86). By measuring apoptosis in cardiomyocytes using TUNEL assays, the group further provided

evidence that a lack of eNOS protects mice against DOX-induced cardiac dysfunction, at least in part by preventing cardiac cell death via apoptosis.

While the above evidence lends support for eNOS's role in anthracycline semiquinone-dependent cardiac damage, eNOS (along with inducible nitric oxide synthase (iNOS) and neuronal nitric oxide synthase (nNOS)) play important roles in the regulation of vascular tone by producing nitric oxide (83). This may be disrupted if anthracyclines like DOX compete with L-arginine as substrates for eNOS. Consistent with this view, as DOX concentrations increase in cells, eNOS activity becomes devoted to semiquinone and superoxide generation rather than nitric oxide production (68, 69). This would result in vasoconstriction of blood vessels in the heart, which may negatively affect heart health under stress. In rabbits, the level of systemic nitric oxide decreases considerably after the administration of DOX (87). In humans, endothelial-dependent and -independent vasodilation were found to decrease considerably after DOX treatment (along with a significant decrease in serum nitrate levels (87)), consistent with inhibition of eNOS-mediated oxidation of L-arginine. Redox cycling, a term used when there is no net increase in the semiquinone because it is continuously reoxidized by molecular oxygen, would exacerbate the inhibition of L-arginine oxidation by eNOS in the presence of anthracyclines, since the spontaneous re-oxidation of the semiquinone would provide a continuous replenishment of the quinone substrate. Moens *et al.* have argued that the uncoupling of eNOS is known to be a major contributor in pressure-overload induced heart failure (88).

The role of iNOS in DOX semiquinone production and DOX-induced cardiotoxicity has been more controversial. It has been suggested that iNOS may elicit cardioprotective effects due to the production of nitric oxide (89), while death-promoting effects may be caused by its facilitation of peroxynitrite formation. Peroxynitrite formation is the result of a reaction between

nitric oxide and superoxide radicals (68, 69), which can have DNA damaging effects (90). The role of nNOS in DOX-mediated cellular toxicity is also unclear. One study reported no change in myocardial nNOS transcript levels upon administration of DOX (91). Nevertheless, it is suggested that the enzyme catalyses the one-electron reduction of DOX to the semiquinone form (92).

Other enzymes such as xanthine dehydrogenase (XDH) (93, 94), NADPH dehydrogenase (NQO1) (94), and NADPH oxidase (NOX) (94) have been implicated in anthracycline-dependent oxygen radical generation, but they may or may not directly catalyse anthracycline semiquinone formation. For example, NQO1 is suggested to contribute to oxygen radical formation during anthracycline treatment (94) and is thus an important factor in treatment related cardiotoxicity; however, it is known to catalyse two-electron reductions and is even suggested to inhibit one-electron reductions, such as semiquinone formation (95). The NOXs have also been examined for their roles in DOX-related superoxide production and cardiotoxicity. Gilleron *et al.* demonstrated that DOX-activated NOXs contributed to superoxide formation and oxidative-stress leading to apoptosis in rat cardiomyoblasts. Moreover, inhibition of NOXs by diphenyliodonium and apocynin strongly reduced DOX-induced oxygen radicals, as well as cell death (96). Supporting this view, experiments using a *Nox2* knock-out model revealed that the left ventricular ejection fraction (8 weeks after DOX treatment) was considerably higher in *Nox2*-deficient mice compared to wild-type mice (97). Interestingly, the study also showed that the adverse effects of DOX could be diminished by treatment with the vasodilator losartan (97).

3.5.2: Tumor Toxicity via Anthracycline Semiquinone Metabolites

Generally, the semiquinone metabolite of anthracyclines is thought to be more cytotoxic to tumors than the unmodified quinone. Although the ability of DOX to generate reactive

oxygen species in tumors has been well demonstrated, several studies suggest that its ability to create covalent modifications or adducts in cellular materials accounts for the enhanced effect of the semiquinone metabolite relative to the quinone (67, 98). Cummings *et al.* have suggested that within tumors, DOX metabolism is mostly impacted by NADPH-cytochrome P450 reductase (99).

3.5.2.1: NADPH-Cytochrome P450 Reductase (CPR)

NADPH-cytochrome P450 reductase (CPR) is a membrane-bound enzyme localized to the endoplasmic reticulum (100). It is an essential reductase, as its ability to transfer electrons is required for the functionality of most cytochrome P450 enzymes (101) and heme oxygenases (102). It is also an important component of xenobiotic metabolism and plasma cholesterol homeostasis (102). CPR catalyses the conversion of DOX to its semiquinone form and it is suggested that this transformation can occur in a variety of benzanthroquinones, including DNR and DNROL (70). Bartoszek observed that MCF-7 cells treated with varying doses of DOX in the presence of exogenous purified rat P450 reductase and NADPH were considerably more sensitive (6-fold) to cell killing than cells incubated with drug alone (67). Additional experiments showed that the potentiation of the drug was abrogated when the experiment was performed by incubating the drug with P450 reductase and NADPH for an hour at physiological temperatures *prior* to administration to tumor cells. This suggested that the semiquinone species is short-lived, becoming reoxidized back to the quinone form even in the absence of cells (103). The enhanced biological effect in this study was not associated with altered drug uptake, since the concentration of isotopically labeled DOX in the cell was unaffected by the presence of the reductase (67, 103). It was also observed that the one-electron reduction of DOX catalysed by P450 reductase took place only under aerobic conditions (103) and involved extensive NADPH

consumption (67). The oxygen requirement in the one-electron reduction was explained using quantum calculations, which showed that the quinone anthracycline (when in complexes with singlet oxygen) is a better electron acceptor than the free quinone (104) (reviewed in 67).

The possible role of P450 reductase, oxygen radical formation, and lipid peroxidation in tumor cell killing by anthracyclines was recently assessed (67). Malondialdehyde (MDA) levels, a measure of lipid peroxidation in cells, were only slightly (but not significantly) increased in the presence of both DOX and P450 reductase relative to the drug alone. Moreover, the correlation between MDA levels and drug cytotoxicity was quite weak (67). A variety of radical scavenging agents have been previously shown to affect DOX induced oxygen radical production (105, 106), but Bartoczek *et al.* observed that oxygen radical formation was not responsible for tumor cell death in the presence of CPR (103). These researchers and others further showed that CPR caused a significant increase in the amount of irreversible associations between radiolabeled DOX and both cellular proteins and DNA and that the formation of these adducts correlated with drug cytotoxicity (67, 103). These findings thus suggested that CPR's ability to enhance DOX cytotoxicity appeared to be related to the formation of alkylating metabolites rather than augmented redox cycling.

Kostrzewa-Nowak *et al.* investigated the effects of human liver CPR on reductive activation of DOX during the treatment of human promyelocytic HL60 cells and multi-drug resistant derivatives of these cells overexpressing either P-glycoprotein or MRP-1 (98). The study involved the reductive activation of DOX by CPR and NADPH extracellularly, followed by treatment of the cells, allowing the metabolite to diffuse into the cells. Their findings indicated that NADPH is a necessary cofactor for CPR-dependent reductive conversion (semiquinone formation) and this formation does not occur at NADPH concentrations below 500

M. Only at high NADPH concentrations was CPR effective at both reductive conversion of DOX and achieving between a two and three-fold increase in toxicity in both the DOX-sensitive and DOX-resistant human leukemia cell lines (98). The study was conducted at clinically relevant doses of DOX, since DOX levels can reach 1-2 μ M in the plasma of patients receiving treatment (14). Interestingly, since drug potentiation occurred in cells overexpressing drug transporters capable of exporting DOX out of the cells, it was further proposed by Kostrzewa-Nowak *et al.* that the reactive metabolite is able to bind to cellular targets and escape MDR protein pumps (98). These investigators also showed that the addition of superoxide dismutase, an oxygen radical-scavenging enzyme, abrogated reductive conversion of DOX by CPR in the presence of sufficient NADPH (98). Given that superoxide dismutase is known to scavenge singlet oxygen (107), this is consistent with the proposal by Tempczyk *et al.* that quinone anthracyclines interacting with singlet oxygen are better electron acceptors than free quinones and that a DOX-singlet oxygen complex may be required for semiquinone formation (104).

As recognized by Kostrzewa-Nowak *et al.*, there is some evidence that contradicts CPR's potential role in DOX activation. Niitsu *et al.* have also shown an inverse relationship between CPR expression and DOX cytotoxicity (108). However, Kostrzewa Nowak *et al.* address this discrepancy by arguing that the reductive activation of DOX by CPR "could be influenced by many factors such as bioavailability of NADPH, levels of other competing metabolic enzymes, and tissue-specific antioxidant defence systems" (98).

Another study supports the notion that reductive conversion of DOX increases its cytotoxic effect in tumor cells (109). In this study, two acute lymphoblastic leukemia (ALL) cell lines were characterized for their ability to reduce DOX to the semiquinone. One cell line was sensitive to DOX (EU3-Sens) and the other was resistant (EU1-Res). The levels of transcripts

and activities for a variety of enzymes and cofactors required for the reductive conversion of DOX were monitored in the cell lines and it was found that the DOX-sensitive cells exhibited lower intracellular quinone levels and higher NADPH levels than the DOX-resistant cells. This is consistent with CPR-dependent reductive conversion of the drug to the semiquinone form. It was also determined that the DOX-resistant cells had higher levels of the superoxide-generating enzyme NADPH oxidase 4 (NOX4) and lower glucose-6-phosphate dehydrogenase (G6PDH) expression. The latter enzyme is responsible for regenerating cellular NADPH. The authors suggest that these changes in NOX4 and G6PDH expression favour the redox cycling and reduced cytotoxicity of DOX in DOX-resistant cells. Interestingly, these investigators conducted similar experiments at lower DOX concentrations (100 nM) and found increased quinone accumulation in the drug-sensitive cell line relative to the drug-resistant cell line (109).

Given all of the above investigations, there is strong evidence for DOX semiquinone formation in both cardiac tissue and tumor tissue and this is associated with its increased cytotoxicity. The mechanism by which the semiquinone metabolite promotes cell death appears to differ considerably between cardiac and tumor tissue. Oxygen radical production resulting from DOX, the semiquinone metabolite, and redox cycling between the two, occurs in cardiomyocytes and is important in cardiotoxicity (105, 110). In contrast, redox cycling and oxygen radical formation does not appear to contribute to tumor toxicity. Rather, alkylation of cellular targets by the semiquinone metabolite appears to promote increased DOX cytotoxicity (67, 103). The enzymes responsible for anthracycline semiquinone formation also appear to differ considerably between tumor and host tissues, especially since malignant transformation itself has been shown to be associated with increased levels of CPR (111). The differences between host and tumor tissue with respect to the enzymes involved in semiquinone formation

as well as the mode of toxicity of the semiquinone metabolite may highlight some inherent differences between healthy and malignant tissue, the latter being considerably more genetically unstable. This genetic instability may help to explain why reductive conversion seems to be a requirement for killing tumor tissue but redox cycling may be sufficient for damaging cardiac tissue, as tumor cells may be more adaptable to increased oxygen radical production. Moreover, DOX-resistant tumor cells may have alterations in their cellular physiology that favor redox cycling. These differences in anthracycline metabolism between host and tumor tissue may present unique opportunities to improve tumor killing by anthracyclines, while sparing host toxicities.

3.6: Reductive Deglycosylation of Anthracyclines (Deoxyaglycone Formation)

The third, and minor, route of anthracycline metabolism involves the reductive deglycosidation of anthracyclines to 7-deoxyaglycone metabolites. The 7-deoxyaglycone form of an anthracycline is essentially the drug with the sugar moiety removed. The formation of 7-deoxyaglycone requires a two-electron reduction and absolutely requires an anaerobic environment and NADPH (27). It is generally believed that the formation of the 7-deoxyaglycone metabolite inactivates the parent molecule. However, the 7-deoxyaglycone metabolite has been implicated in the production of ROS by intercalating with the inner mitochondrial membrane due to the increase in lipid solubility that accompanies the loss of the sugar moiety (14, 112). This intercalation into membranes has also been thought to cause a form of benign acute toxicity in the human myocardium via ROS production (32), although the cardiotoxicity is not to the extent of that observed for the parent molecule. For DOX, the 7-deoxyaglycone metabolite can be produced from both the parent DOX molecule and also from the metabolite DOXOL. Considering that the four main anthracyclines (DOX, EPI, DNR, and

IDA) share very similar structures it can be inferred that the formation of 7-deoxyaglycone would occur in similar ways for all four anthracyclines. Formation of the 7-deoxyaglycone metabolite occurs via three enzymes: NAD (P)H Quinone Oxidoreductase 1 (NQO1), Cytochrome P450 Reductase (CPR), and Xanthine Dehydrogenase (XDH).

3.6.1: NQO1

NAD (P)H Quinone Oxidoreductase 1 (NQO1), also known as DT-diaphorase, is an NADH- or NADPH-dependent enzyme (99). The main role of NQO1 is to detoxify quinones via a two-electron reduction (27). Other roles that NQO1 fulfills include the maintenance of antioxidants (113), the stabilization of p53 (114, 115), acting as a superoxide scavenger (116) and a 20S proteasome gatekeeper (117). However, NQO1's ability to perform a two-electron reduction of anthracyclines to form the deoxyaglycone metabolites depends heavily upon the physiological environment at the tumor site (99), with the deoxyaglycone only forming under hypoxic conditions (118). In this way the harmful semiquinone metabolite is bypassed in favour of the inactive deoxyaglycone metabolite. NQO1 has also been implicated in the formation of semiquinone radicals of anthracyclines in an aerobic environment (27, 94), noting, however, that the deoxyaglycone metabolite is the predominant metabolite under anaerobic conditions (99). Interestingly, NQO1 is predominantly found in the cytosol of cells (99), with the highest levels of the enzyme found in cardiovascular tissues and the liver (119).

NQO1 expression is induced by many xenobiotics via the Keap1/Nrf2/ARE pathway (120). While Nrf2 is a critical transcription factor involved in cellular protection from toxic xenobiotics, it is kept at low levels under unstressed conditions by Keap1 (121). The promoter region of the NQO1 gene contains multiple antioxidant response elements (AREs) and a xenobiotic response element (XRE) (119, 120) that bind Nrf2 and regulate NQO1 expression.

Nrf2 has been implicated in chemotherapy drug resistance *in vitro*. Increased Nrf2 expression and activity have been associated with increased NQO1 levels and DOX resistance in both A2780 ovarian tumor cells and MCF-7 breast cancer cells (54, 122, 123). While inactivation of Nrf2 in these cell lines can restore DOX cytotoxicity (124), it has been shown in rabbits that continuous treatment with anthracyclines does not elevate Nrf2 levels, while NQO1 levels are down-regulated (125).

Recently, a number of *NQO1* polymorphisms have been identified and their relationship to patient survival after anthracycline chemotherapy was assessed. One such *NQO1* polymorphism (C609T) results in a Pro187Ser substitution, which results in the increased ubiquitination and degradation of the protein (126). Due to the degradation of NQO1, there is an impairment in ROS detoxification and reduced survival after chemotherapy (127). If a patient is homozygous for the T allele (Ser substitution), the enzyme activity appears to be reduced to only 2% of the wild type enzyme (homozygous for the C allele) (126, 128, 129). Although the T allele has not been found to be significantly related to patient survival for all common cancer sites (127, 130, 131), there is a clear trend toward an increased overall cancer risk associated with this polymorphism (130). Moreover, this lack of significance could be attributed to the small sample size associated with the low frequency T allele (130, 131). Interestingly, inhibition of NQO1 by dicoumarol was found to be associated with an increase in DOX-induced cardiotoxicity, suggesting NQO1 may play a protective role against DOX-induced cardiotoxicity (132).

3.6.2: CPR and XDH

Cytochrome P450 Reductase (CPR) is also dependent on NADPH (99). CPR can catalyze both redox cycling in an aerobic environment and 7-deoxyaglycone formation in an

anaerobic environment (99). It has been shown that DOX is stoichiometrically converted to the inactive 7-deoxyaglycone metabolite by CPR, and, similar to NQO1, this formation was completely abolished in an aerobic environment (99). CPR is found predominantly in the liver; however, it can be found in other organs such as the lung or the kidney (133). Interestingly, the anthracyclines DNR and DOX have been shown to suppress the activity of CPR in rabbit hepatocytes *in vitro* (134).

Xanthine Dehydrogenase (XDH) is less effective than both NQO1 and CPR at generating 7-deoxyaglycone metabolites of anthracyclines (99). Consequently, its role in this process is less studied. XDH has also been implicated in the formation of the semiquinone radical (94).

3.7: Strategies to Improve Clinical Response and/or Reduce Clinical Toxicity to Anthracyclines

As mentioned previously, one of the most prevalent and serious side effects that limits the use of anthracyclines is their cardiotoxicity. This is particularly the case for breast cancer patients undergoing anthracycline-based adjuvant or neoadjuvant chemotherapy (135). To reduce the incidence of cardiac toxicity, longer infusion rates are employed to reduce peak plasma levels of anthracyclines. Their cumulative doses are also closely monitored. Nevertheless, these strategies do not completely eliminate the risk of cardiotoxicity (136). Reduced cardiotoxicity with anthracyclines using liposomal formulations of DOX or DNR to selectively target tumor tissue has shown some efficacy in a clinical setting (137). Another method for preventing anthracycline-induced cardiotoxicity involves the use of pharmacological cardioprotective agents, such as dexrazoxane (138), ascorbic acid (139), and an engineered bivalent neuregulin (140). To date, the only agent in this class that has been approved for use

and has shown significant clinical efficacy is the iron chelator dexrazoxane (DEX) (138, 141). While other pharmacological cardioprotective agents have been tested in an *in vitro* setting, their use clinically is uncertain at this time.

Two approaches have been used to improve the therapeutic index of anthracyclines. One involves altering the metabolism of the drugs in order to enhance their cytotoxic effects on tumors by enhancing their therapeutic mechanism of action. This could include nucleic acid intercalation, topoisomerase II inhibition, iron mediated generation of ROS, and other processes that could lead to improved killing of tumors *in vivo* (14). A second approach is to reduce the systemic toxicity of anthracyclines by further chemical modification of anthracyclines or by selectively inhibiting off target effects of the drug and its metabolites, such as cardiotoxicity. Examples of these approaches are illustrated below.

3.7.1: Modulators to enhance the tumor cytotoxicity of anthracyclines

3.7.1.1: AKR and CR Inhibitors

As mentioned previously, the 13-hydroxylation of anthracyclines by AKRs substantially reduces their tumor cytotoxicity (37, 39, 40). Thus, blocking the formation of hydroxylated metabolites could improve the efficacy of anthracyclines. Inhibiting AKRs with agents such as 2-hydroxyflavanone (48) or β -cholanolic acid (54, 55) have been shown to increase anthracycline cytotoxicity in anthracycline-resistant cell lines overexpressing AKRs, but it is unclear whether a similar improvement in clinical response to anthracyclines would be realized in patients with tumors that have intrinsic or acquired resistance to these agents. The semisynthetic flavonoid 7-mono-O- (-hydroxyethyl)-rutoside, commonly known as mono-HER, is known to effectively inhibit CBR1. However, unlike the AKR inhibitors, mono-HER has been documented in a phase II clinical trial to protect metastatic cancer patients from DOX-induced cardiotoxicity (142).

This may be through its ability to reduce production of the cardiotoxic metabolite DOXOL. Mono-HER has also been shown to potentiate the cytotoxicity of DOX in human liposarcoma cells by reducing NF- κ B activation and promoting DOX-induced apoptosis (143). *In vitro* and *in vivo* experiments have shown that mono-HER does not interfere with the antitumor effect of DOX. Interestingly, high doses of mono-HER (>1500 mg/m²) can augment DOX's anti-tumor effect, while considerably lower doses are required to achieve mono-HER's cardioprotective effect (68).

3.7.2: Strategies to Reduce the Systemic Toxicity of Anthracyclines

3.7.2.1: Iron Chelators (Dexrazoxane)

Iron is known to potentiate the toxicity of anthracyclines by transforming relatively safe species like O₂ • and H₂O₂ into much more reactive and toxic hydroxyl radical (OH•) or iron-peroxide complexes that have the capacity to damage DNA, proteins, and lipids (8, 144). It has also been proposed that the redox cycling of the quinone moiety would allow anthracyclines to increase cellular levels of iron by mobilizing ferritin, a ubiquitous intracellular protein that stores and releases iron (145). In addition, doxorubicin treatment has been shown to result in the preferential accumulation of iron inside the mitochondria of cardiomyocytes (146). This results in amplification of iron-mediated oxidative stress (147, 148). Moreover, doxorubicin-induced DNA strand breaks and changes in gene expression that lead to defective mitochondrial biogenesis and ROS formation (and subsequently cardiotoxicity) appear to be reduced in mice having cardiomyocytes possessing *Top2b* deletion mutations, suggesting that cardiotoxicity by doxorubicin involves the action of topoisomerase II (149). Perhaps one of the best pieces of evidence that iron plays a pivotal role in the ROS-mediated toxicity of DOX comes from numerous studies showing that the iron chelator dexrazoxane (DEX) can effectively block

anthracycline cardiotoxicity (138). DEX has been repeatedly shown to mitigate anthracycline toxicity, and is approved for clinical use (150-152). Marty *et al* have shown in a randomized phase III study of 164 breast cancer patients that in comparison to patients receiving an anthracycline alone, patients treated with both an anthracycline and DEX experienced significantly fewer and less severe episodes of congestive heart failure, without affecting the tumor response rate (116). Other antioxidants have also been shown to reduce both the ROS generation and toxicity of anthracyclines, but their current use remains limited (138).

3.7.2.2: Statins, B Blockers, ACE inhibitors, and COX inhibitors

Recent studies have shown that several statins such as lovastatin (153), β blockers such as Nebivolol (154), ACE inhibitors (e.g. Enalapril) (155, 156) and COX inhibitors demonstrate notable cardioprotective effects, when used in conjunction with DOX (157). Interestingly, in a recent review of randomized trials and observational studies, where a prophylactic intervention was compared with a control arm in patients with a normal ejection fraction and no past history of heart failure, the authors demonstrated that prophylactic treatment with DEX, a β -blocker, a statin, or angiotensin antagonists all can reduce cardiotoxicity (157). Statins have been suggested as an alternative cardioprotective strategy for anthracycline treatments (158). In mice, DOX increases the cardiac mRNA levels of B-type natriuretic peptide, interleukin-6 and connective tissue growth factor, while lovastatin appeared to counteract these anthracycline-induced cardiac stress responses (159). Zofenopril, an ACE inhibitor, inhibits cardiotoxicity in rats; however, it is unclear whether the mechanism is direct ACE inhibition or another off target cardioprotective effect (160). The same can be said for statins, β blockers and cyclooxygenase-2 inhibitors. The mechanisms by which these agents prevent cardiotoxicity are not well understood, limiting their use and study as agents to combat anthracycline cardiotoxicity (154). Moreover, no randomized

studies have been published comparing the cardioprotective efficacy of statins, β blockers, ACE inhibitors, or COX inhibitors relative to DEX.

3.7.2.3: Glucuronidase-Mediated Release of Anthracycline Prodrugs

Another mechanism for reducing the systemic toxicity of anthracyclines, including cardiotoxicity, is to restrict drug action to tumor sites. For example, a series of glucuronide prodrugs have been synthesized that render DOX nontoxic to host tissues, since the hydrophilic glucuronide group added to DOX prevents entry across hydrophobic cellular membranes. However, the high levels of β -glucuronidase in tumors then permits the release of cytotoxic DOX and tumor-specific cell killing (161-163). While such prodrugs have shown promise in pre-clinical models (164, 165), none, to our knowledge, have yet to enter clinical trials in humans. This may be due to wide variations in β -glucuronidase levels amongst patient tumors, necessitating screening of patient tumors for high glucuronidase expression (166).

3.7.2.4: Fullerenol

The chemical modification of fullerenes (specifically, the polyhydroxylation of C60 nanoparticles) results in C60 (OH)_x structures with differing degrees of antioxidant activity in an aqueous environment (167). One particular fullerene (fullerenol) was able to protect rat hearts from DOX-induced cardiotoxicity (168). It has been proposed that fullerenol protects cardiomyocytes by acting as a ROS scavenger and/or by removing free iron through the formation of a fullerenol-iron complex (168). Previous studies have shown that application of DOX to Wistar rats causes damage to the heart and baroreceptors, which results in diastolic dysfunction characterized by increased left ventricle end-diastolic pressure (169). Fullerenol

attenuated these DOX-induced heart disturbances (168). Fullerenol was also able to successfully treat DOX-induced nephrotoxicity (170), pulmototoxicity (171), and hepatotoxicity (172) in rats.

3.7.2.5: Synthesis of Non-Cardiotoxic Anthracyclines

An alternate strategy for combating anthracycline-induced cardiotoxicity is to synthesize anthracyclines with reduced capacity for cardiotoxicity. For example, a prodrug of DOX (aldoxorubicin) can be administered at significantly higher concentrations in patients, without acute cardiotoxicity. Moreover, recent findings further suggest that aldoxorubicin exhibits stable levels in blood without accumulation in body compartments such as the heart. This may explain why the drug is significantly less cardiotoxic than DOX (173). The DOX analog GPX-150, modified at two sites to reduce the formation of cardiotoxic metabolites or ROS, has shown promise in a recent phase I trial, where it was administered safely to patients with acceptable toxicity and no cardiotoxicity (174). Another anthracycline with protein kinase C-activating properties (AD 198) has also shown significant anti-tumor activity. However, unlike DOX, little ventricular damage was observed in mice administered the agent (175).

3.8: Concluding Remarks

In the case of anthracyclines, a knowledge of the metabolism of these drugs has provided significant insight into how they exert their anti-tumor effects and their toxic side effects on the host. Some anthracycline metabolites facilitate the ability of these drugs to combat the growth of tumor cells, while others suppress cytotoxicity. The hydroxylation of anthracyclines is of particular interest, since the hydroxylated metabolites have considerably reduced antitumor activity, but substantially increased cardiotoxicity. By identifying the precise enzymes that play a role in anthracycline metabolism, it has been possible to identify novel chemical agents that

can augment the anti-tumor effects of anthracyclines and/or prevent negative side effects within the host, including cardiotoxicity, nephrotoxicity, and hepatotoxicity. While successful new strategies to improve the therapeutic index of anthracyclines have recently been identified, it will only be through future clinical trials in multiple, independent cohorts of patients that the true efficacy of these strategies will be known. For one such agent (dexrazoxane), its incorporation into standard clinical practice clearly appears to be on the horizon.

3.9: CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

3.10 Acknowledgements

Declared none.

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Chapter 4

Background/Rationale for Studies on Estrogen Signalling Pathway

4.1: Introduction

To study the phenomenon of acquired chemotherapy resistance, a series of chemotherapy resistant cell lines were developed. Briefly, this was accomplished by growing MCF-7 breast cancer cells in increasing concentrations of chemotherapy agents beginning at a concentration approximately 1,000 fold below the IC₅₀ for each chemotherapy agent (1–3). Cells would be maintained at this concentration, termed “dose 1”, for a period of 2-3 passages or 2 weeks (2). The concentration would then be increased by 3 fold (2). This process would be repeated until the cells were no longer able to adapt to the increasing concentrations of chemotherapy drug and did not grow (2). This final dose selection was termed the terminal selection dose (2). To control for possible changes in gene expression due to prolonged propagation of the cells rather than exposure to chemotherapy, co-culture control cells were propagated in parallel which were never exposed to chemotherapy (2).

Microarray analysis was performed on the terminally selected cells comparing mRNA expression levels (2). Analysis of the data indicated significant changes in gene expression of over 3,000 genes (2). Among the largest changes, between the MCF-7_{DOX2-12} (terminal selection dose) cells and co-culture control cells, were members of the aldo-keto reductase family, specifically AKC1C3 and AKR1B10 (1, 3). Aldo-keto reductases (AKR) comprise a superfamily of proteins. All members of the AKR enzyme superfamily function by reducing ketones and aldehydes into secondary and primary alcohols (4). They perform this action in an NADPH-dependent manner (4). As of July 24, 2017, the superfamily contained more than 180 members, sub-categorized into 15 families. AKRs are classified into families based on sharing a minimum of 40% protein sequence identity and into sub-families by sharing more than 60% sequence identity (4). Individual members are identified using a nomenclature where the proper

name begins with AKR, designating the superfamily of proteins, followed by a number designating the family, then a letter to denote the sub-family and finally a number designating the individual member (e.g. AKR1C3 (for the gene) or AKR1c3 (for the protein)) (4). The AKR1 family is the largest of the 15 families and is one of three families corresponding to the mammalian AKRs (4).

Most AKRs are monomeric proteins ranging in size from 34-37 kDa, however some multimeric forms have been identified. These latter proteins are members of the AKR2, AKR6 and AKR7 families (4). Of the 25 known crystal structures of AKRs, all have been shown to exhibit an alternating (α/β)₈ barrel motif. The folded form of the enzyme contains binding sites for both the substrate as well as for the co-factor (NADPH), both of which converge at the active site of the enzyme (4). While the co-factor binding site and the active site are highly conserved among members of the AKR superfamily, the substrate-binding sites show high levels of variation in the sequence of amino acids comprising the connecting loops between the helices and the β sheets of the enzyme (4).

The AKR1 family contains six sub-families, the largest of which is the AKR1C subfamily (4). AKR1C members primarily function as steroid-metabolizing enzymes. Molecules of the AKR1C1-1C4 all share more than 86% sequence identity (4). AKR1C3, also known as 17 β -hydroxy-steroid-dehydrogenase type V (17 β -HSD) or 3 α -hydroxy-steroid-dehydrogenase type I (3 α -HSD), is involved in estrogen metabolism (4). AKR1C3 is responsible for the conversion of steroid molecules into their active forms; in particular, it converts androstenedione into testosterone and estrone (E1) into estradiol (E2). In addition to their ability to metabolize steroid molecules, the AKR1C family has been shown to metabolize chemotherapeutic agents (4). AKR1B10 and AKR1C2 have been shown to convert the anti-tumour agent doxorubicin into

doxorubicinol (4). AKRs have been implicated in a number of other biological processes, including but not restricted to: carcinogenesis through the activation of polycyclic aromatic hydrocarbons (AKR1C3-AKR1C4), and detoxification of nicotine carcinogens (AKR1C1, AKR1C2, and AKR1C4), aflatoxin (AKR7A family members), and reactive aldehydes (AKR1A1, AKR1B1, AKR1C1, AKR1C4 and AKR7A2) (4).

AKR expression is generally regulated in response to environmental signals such as osmotic pressure and the production of reactive oxygen species (ROS) (4). This regulation occurs as a response of transcriptional elements found in the promoter region of the genes. As of 2007, three different environmental response elements have been identified in the AKR promoter region. An osmotic response element has been identified in the 5' flanking region of AKR1B1 (4). AP-1 consensus sequences have been identified in the AKR1B1 promoter as well as in the promoters of members of the AKR1C family (4). However, these sites have yet to be demonstrated as functional (4). As it has been shown, that AP-1 sites are embedded within many antioxidant response elements (AREs), cellular responses to ROS may, in part, be mediated via the AP-1 sites (4). The expression of the AKR1C1-1C3 enzymes are all regulated by AREs through Nrf2-Keap-1 complexes (4).

The AKR1C1-AKR1C4 enzymes have all been shown to interact with a variety of substrates *in vitro*. In the case of AKR1C3, its structure may be related to its multi-specificity for substrates (5). AKR1C3 provides a well-described example of plasticity. It is capable of metabolizing several different steroid molecules, including 4-androstenedione and estrone (E1) (6). These molecules are steroid precursors to testosterone and estradiol (E2) respectively.

Estrogen synthesis is of particular interest in breast cancer studies, due to the effects of estrogen on breast epithelial cell proliferation (4, 7) and survival (8). Estrogen signaling begins

with the binding of an estrogen molecule to one of two receptors, estrogen receptor α (ER α or ESR1) or estrogen receptor β (ER β or ESR2). Both receptors belong to the superfamily of steroid nuclear receptors and have a common structural makeup (8). Binding occurs when an estrogen molecule diffuses across the membrane and enters the cytosol (9). The estrogen molecule then binds to either cytoplasmic or plasma membrane-bound receptor. Upon binding, the receptor undergoes phosphorylation via cyclin A/CDK2 or TFIIH cyclin dependent kinase. Although the best defined action of estrogen receptors is to function as a nuclear transcription factor (9), it has recently been demonstrated that E2 can affect cellular processes more quickly than is possible by affecting gene transcription. In some cases, these effects may be mediated by membrane bound estrogen receptors or other surface receptors such as the G-protein coupled receptor 30 (GPR-30) (9, 10). Upon phosphorylation of membrane bound receptor α , the MAPK (growth promoting), or PI3K (survival) pathways can be activated by a mechanism not requiring gene transcription (11–14). Another estrogen signaling pathway is the classical genomic signaling pathway. In this situation, phosphorylated receptors dimerize to form active complexes, enter the nuclei of cells, and bind to estrogen response elements (EREs) in the promoter regions of specific genes (9). Once bound to the ERE, the active ER complex will recruit other transcription factors and activate transcription of associated genes (9).

ER α has long been used as a prognostic biomarker in cancer, in particular for female cancers. ER α expression is generally associated with good prognosis in breast cancer as many therapies exist to target this receptor or the pathways upstream of the receptor (15). Recently, ER β has emerged as a possible new prognostic biomarker in breast cancer (15). In histological studies, ER β was shown to be a marker of good prognosis in post-menopausal women; this included patients diagnosed with triple negative breast cancer (i.e. lacking ER α) (15). The

importance of studying ER α and ER β , their interactions with each other, and how they influence the development and prognosis of breast cancer, has been recognized for many years (16–19). In both breast cancer and prostate cancer, steroid hormones have been shown to protect cells from undergoing apoptosis. This was demonstrated by removing testosterone and estrogen from prostate and mammary epithelial cells, respectively (8). When they are grown in the absence of steroid hormones, the cells underwent apoptosis. Previous studies have shown that MCF-7 cells pretreated with estrogen show resistance to killing by TNF- α and other chemotherapeutics, including the ER α antagonist tamoxifen (20). The ability of estrogens to interfere with apoptosis is generally attributed to their ability to promote overexpression of the apoptotic suppressor proteins BCL-2 or BCL-XL. Although BCL-2 does not contain an ERE in the promoter region of the gene, it does contain one within the coding region (21). In addition, the anti-apoptotic effects of estrogens have been shown to involve the mitogenic MAPK/ERK pathway as well as the anti-apoptotic PI3K/AKT pathway via activation of membrane-associated ER α . In MCF-7 cells, it has been shown that estrogens have differential effects on the expression of the BCL family of proteins; treatment with estrogens increases the expression of BCL-2, while reducing expression of BCL-xL (22, 23). Since both of these proteins have anti-apoptotic effects, this phenomenon is generally regarded as being paradoxical in terms of promoting cell survival. In addition to having anti-apoptotic effects, estrogens have also been shown to stimulate the growth of MCF-7 cells (24). This was shown in previous studies by growing cells in serum depleted of all steroids. These cells showed a marked reduction in cell proliferation, possibly by preventing estrogen-dependent activation of the MAPK pathway or by permitting apoptosis resulting from the absence of estrogen dependent BCL-2 expression (24–26). The latter was disproven using live

microscopic techniques which showed that in the absence of estrogens, the cells were still able to move and act as they would normally, but at a reduced rate.

Most hormone-dependent cancers rely on locally synthesized hormones for signaling as opposed to systemic steroids (24). In many cases, the tumours themselves act as the source for these growth hormones. This is of particular advantage for the cancer cells since it is more energetically favorable to use an intracrine system of signaling, which requires a lower level of hormones as opposed to endocrine or paracrine systems (27). The two latter systems require higher levels of hormones to achieve the same results on signal activity, since the signal gets diluted as it diffuses further away from the source of synthesis (27).

4.2: Hypothesis and Specific Aims of Study 1

I postulate that the increase in AKR1C3 expression will result in higher levels of serum estradiol. This will result in one of two possibilities: 1) An increase in estrogen signaling will resulting in enhanced survival and increased growth of the cells, or 2) increased estradiol levels that will result in a negative feedback in turn reducing the ER α levels and resulting in a down regulation of the growth-promoting signal. Based on the above hypotheses, the primary aims of this thesis are to:

Aims:

- 1) Assess AKR1C3 protein levels in wildtype and anthracycline-resistant MCF-7 breast tumour cells,
- 2) Determine if overexpressing AKR1C3 in wildtype MCF-7 cells impart resistance to anthracyclines,

- 3) Use siRNA approaches in MCF-7_{DOX2-12} cells to reduce levels of AKR1C3 and AKR1B10 and determine the effect on resistance to doxorubicin
- 4) Assess the ability to convert estrone to estradiol in MCF-7 anthracycline-resistant and AKR1C3-transfected cells,
- 5) Assess the estrogen receptor signaling activity in MCF-7 Anthracycline-resistant cells by assessing expression levels of BCL-2 and Cyclin D1 as indicators of genomic signaling,
- 6) Assess the growth rate of MCF-7 Anthracycline resistant and AKR1C3 transfected cells to confirm the reduced rate of growth in anthracycline resistant cells,

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Chapter 5

Alterations in estrogen signalling pathways upon acquisition of anthracycline resistance in breast tumor cells

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SC authored the paper and conducted a majority of the experimentation. BG generated cloning vectors and AKR expression plasmids. AMP participated in the conception, outline and drafting of the manuscript. All authors read and approved the manuscript.

5.1: Abstract

Intrinsic or acquired drug resistance is a major impediment to the successful treatment of women with breast cancer using chemotherapy. We have observed that MCF-7 breast tumor cells selected for resistance to doxorubicin or epirubicin (MCF-7_{DOX2} and MCF-7_{EPI} cells, respectively) exhibited increased expression of several members of the aldo-keto reductase (AKR) gene family (in particular *AKR1C3* and *AKR1B10*) relative to control MCF-7_{CC} cells selected by propagation in the absence of drug. Normal cellular roles for the AKRs include the promotion of estrogen (E2) synthesis from estrone (E1) and the hydroxylation and detoxification of exogenous xenobiotics such as anthracycline chemotherapy drugs. While hydroxylation of anthracyclines strongly attenuates their cytotoxicity, it is unclear whether the enhanced AKR expression in the above anthracycline-resistant cells promotes E2 synthesis and/or alterations in E2 signaling pathways and whether such changes contribute to enhanced survival and anthracycline resistance. To determine the role of AKRs and E2 pathways in doxorubicin resistance, we examined changes in the expression of E2-related genes and proteins upon acquisition of doxorubicin resistance. We also assessed the effects of AKR overexpression or downregulation or the effects of activators or inhibitors of E2-dependent pathways on previously acquired resistance to doxorubicin. In this study we observed that the enhanced AKR expression upon acquisition of anthracycline resistance was, in fact, associated with enhanced E2 production. However, the expression of estrogen receptor α (ER α) was reduced by 2- to 5-fold at the gene transcript level and 2- to 20-fold at the protein level upon acquisition of anthracycline resistance. This was accompanied by an even stronger reduction in ER α phosphorylation and activity, including highly suppressed expression of two proteins under E2-dependent control (BCL-2 and cyclin D1). The diminished BCL-2 and cyclin D1 expression would be expected to

reduce the growth rate of the cells, a hypothesis which was confirmed in subsequent cell proliferation experiments. *AKR1C3* or *AKR1B10* overexpression alone had no effect on doxorubicin sensitivity in MCF-7_{CC} cells, while siRNA-mediated knockdown of *AKR1C3* and/or *AKR1B10* expression had no significant effect on sensitivity to doxorubicin in MCF-7_{DOX2} or MCF-7_{EPI} cells. This suggested that enhanced or reduced AKR expression/activity is insufficient to confer anthracycline resistance or sensitivity to breast tumor cells, respectively. Rather, it would appear that AKR overexpression acts in concert with other proteins to confer anthracycline resistance, including reduced E2-dependent expression of both an important apoptosis inhibitor (BCL-2) and a key protein associated with activation of cell cycle-dependent kinases (cyclin D1).

5.2: Introduction

Anthracyclines are a class of drugs that are commonly used in adjuvant or neoadjuvant chemotherapy for breast cancer, often in conjunction with other anti-cancer agents (1). Of this class of chemotherapy agents, doxorubicin or epirubicin are most widely used. Anthracyclines are believed to be cytotoxic to tumor cells through three mechanisms: intercalation between strands of DNA/RNA molecules resulting in interference with normal DNA/RNA synthesis in rapidly dividing cells (2, 3), inhibition of topoisomerase II activity (4), and the creation of iron-mediated oxygen free radicals (5, 6).

Despite their clear utility in the clinical management of breast cancer, many factors negatively affect their efficacy when administered to cancer patients. One such factor is the ability of tumors to resist the cytotoxic action of anthracyclines (7). This can occur via two distinct mechanisms. First, some tumors exhibit innate resistance to chemotherapy drugs, such that they do not respond to first-line chemotherapy (often referred to as “primary chemotherapy”) (8). In other instances, patient tumors acquire resistance to anthracyclines and other chemotherapy agents over time. In this latter case, the tumors initially respond partially or almost fully to the administered drugs. However, drug-resistant cells within the tumor cell population survive treatment and continue to replicate, resulting in recurrent disease and disease progression. In some instances, tumors acquire resistance to a wide variety of chemotherapeutic agents, a phenomenon known as multi-drug resistance (7). Chemo-resistant tumors are usually treated with alternative chemotherapy drugs (9, 10) or alternate downstream treatments such as surgery or radiation therapy (10, 11).

One tool used to study the phenomenon of drug resistance is to look at genotypic and phenotypic changes that take place as tumor cells acquire resistance to chemotherapy drugs in the laboratory. We recently established a panel of MCF-7 breast cancer cell lines, which were selected for survival in increasing concentrations of various chemotherapy agents including the anthracyclines (12). Microarray studies comparing parental and anthracycline-resistant cells revealed many changes in gene expression accompanying the acquisition of anthracycline resistance, including increased transcripts for several members of the aldo-keto reductase (AKR) family (13) and decreased transcription of genes for estrogen receptor α (ER α) and BCL-2 (13). The higher levels of expression of AKRs in the above anthracycline-resistant MCF-7 cells relative to drug-sensitive control cells has also been correlated with reduced cellular doxorubicin content, strongly reduced doxorubicin localization to the nucleus, and substantial sequestration of doxorubicin into perinuclear lysosomes (14).

The AKRs are a superfamily of proteins that hydroxylate various endogenous cellular substrates and chemotherapy drugs (reviewed in (15) and (16)). Individual members are identified using a nomenclature method beginning with AKR, followed by a number designating the family, then a letter to denote the sub-family, and finally a number designating the individual member within the sub-family (e.g. *AKR1C3* (for the human gene) or AKR1c3 (for the protein)) (17). The AKR1 family is the largest of the 15 AKR families and is one of three mammalian AKR families (17). AKRs are differentially expressed in various tissues throughout the body. *AKR1C1* and *AKR1C4* transcripts have been shown to be primarily expressed in the liver, intestine, mammary glands, prostate, and lungs (17–19). AKR1c3 is the dominant AKR found in mammary glands. It is also responsible for the hydroxylation of steroid molecules into their

active forms; specifically, it converts androstenedione into testosterone and estrone (E1) into estradiol (E2) (20).

E2 is a potent signaling molecule which is active in host tissues and tumors that are positive for estrogen receptors (21–23). E2 belongs to the E2 family of signaling steroids, which includes the precursor molecules E1 and estriole (E3). Like all steroid molecules, E2 is synthesized from cholesterol (24). The primary source for E2 in physiological systems differs between males and females. In pre-menopausal women, the majority of E2 is synthesized in the ovaries (25, 26) and serves as the primary source of circulating E2. However, in post-menopausal women and in men, synthesized E2 acts in a paracrine fashion in tissues such as the breast (25). E2 synthesis is of particular interest in breast cancer studies, due to the effects of E2 on breast epithelial cell proliferation (20, 25, 27) and survival (28) via phosphorylation and activation of ER α (29, 30). Epidermal growth factor (EGF), a known promoter of cell proliferation is also known to activate ER α phosphorylation (31).

ER signaling has a wide range of effects within cells, including the regulation of transcription (32, 33) and the activation of anti-apoptotic and pro-growth pathways (34, 35). The genes and pathways affected by E2 vary depending upon which of two E2 receptors is activated (ER α or ER β) (36). Both receptors share a common structural homology but differ in size (66 kDa and 56 kDa for ER α and ER β , respectively) (28, 36).

While ER α expression promotes breast tumor growth, it is generally associated with a favorable outcome for breast cancer patients, as many therapies exist to inhibit ER α function or pathways upstream of this receptor (37–39). Recently, ER β has emerged as a possible new prognostic biomarker in breast cancer (40, 41). Most hormone-dependent cancers rely on locally

synthesized hormones for signaling as opposed to systemic hormones (24). In many cases, the tumors themselves act as the source for these growth-promoting hormones. This is of particular advantage for cancer cells, since it is more energetically favorable to use a local autocrine system to promote growth, as opposed to the higher hormone levels required for endocrine or paracrine systems (24).

In this study, we examined, in ER+ MCF-7 breast tumor cells, the effects of selection for anthracycline resistance and/or the altered expression of one or more AKRs on E2 metabolism, E2-dependent signaling pathways, expression of E2-dependent genes, cellular growth kinetics, and cellular survival in the presence of anthracyclines. We postulated that, in addition to effects on doxorubicin catabolism and cellular levels of active doxorubicin, the increased expression of AKRs in anthracycline-resistant or AKR-transfected cells will result in higher levels of E2 production and increased cellular proliferation. In addition, we expected to observe increased expression of E2-dependent genes that promote increased tumor cell survival in the presence of anthracyclines.

5.3: Methods

5.3.1: Culture of wildtype and drug-resistant breast tumour cell lines

The MCF-7 breast cancer cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in high glucose D-MEM medium supplemented with 10% FBS, 100 µg/ml streptomycin, and 100 units/ml penicillin (Hyclone, Mississauga, Ontario) at 37° C in 5% CO₂. For subculturing, cells in T75 Sarstedt flasks were washed once with sterile PBS followed by the addition of 3 ml of a sterile 0.25% Trypsin, 10 mM EDTA solution (Invitrogen, Burlington Ontario). MCF-7 cells were selected for resistance

to increasing doses of either doxorubicin or epirubicin as described previously (12). Cells at the various drug selection doses (1 through 12) were stored for subsequent studies. Cells were named based on the selection process, the selection agent and the selection dose. For example, MCF-7_{DOX2-9} cells represent the second time that MCF-7 cells were selected for resistance to doxorubicin up to selection dose 9. Similarly, MCF-7_{EPI-12} cells represent the first time that MCF-7 cells were selected for resistance to epirubicin up to selection dose 12. The various selection doses employed are described in our previously published study (12). Cells were also selected in the absence of drug to control for genotypic and phenotypic changes due to continued propagation in culture (at identical passage numbers to drug-selected cells). The nomenclature used for these cell lines reflect the absence of drug (co-cultured control or “CC” cells) and the number of passages. For example, MCF-7_{CC-12} cells are cells selected in the absence of drug for an equivalent number of passages as anthracycline-selected cells (selected to dose 12). Drug-resistant cells were maintained in media supplemented with the corresponding selection dose of doxorubicin or epirubicin. They were, however, placed in drug-free medium media for 3–4 days prior to use in experiments.

5.3.2: Dextran charcoal filtering of cell culture media

To remove endogenous steroid compounds and growth factors, D-MEM media supplemented with 10% FBS and antibiotics were treated with 0.5% (w/v) dextran-coated charcoal (Sigma Chemical, St. Louis, MO), and heated to 50°C with constant shaking for 30 min. The charcoal-treated media was then sterile-filtered using a Sarstedt 0.22 µm, 500 ml vacuum filter. Dextran charcoal-treated D-MEM media (DC-DMEM) were supplemented with 10⁻⁷ M E2 for ER signaling experiments, or 10⁻⁷ M estrone (E1) for E1 metabolic experiments (Sigma Chemical, St. Louis, MO).

5.3.3: Assessment of cellular E2 levels

Estradiol (E2) levels in wildtype and drug-resistant cells were determined using a standard enzyme-linked immunosorbent assay (ELISA). Briefly, 5×10^5 cells/well were plated in 6 well plates (Sarstedt), in D-MEM media supplemented with 10% FBS and antibiotics and allowed to adhere overnight. D-MEM media was replaced with dextran/charcoal-stripped D-MEM medium (DC-DMEM) 18 hours after plating. Cells were treated with one of: a vehicle control solution (5% DMSO), 10^{-7} M E1, 10^{-7} M E1 with 5×10^{-9} M letrozole (aromatase inhibitor), or 10^{-7} M E1 (E1) with 0.2 mM β -cholanolic acid (in 5% DMSO) (AKR1c3 inhibitor). Aliquots of media were collected 24h post treatment and stored at -20°C . E2 levels in the media were then assayed using E2 competitive ELISA kits (US Biological Life Sciences) as per the manufacturer's protocol.

5.3.4: Assessment of cellular sensitivity to anthracyclines using clonogenic assays

The sensitivity of the above-described cell lines to various doses of anthracyclines was determined using a clonogenic assay, which monitors the ability of drug-treated cells to form colonies in drug-free semi-solid methylcellulose medium, as previously described in (13). The mean number of viable colonies was determined in 12 randomly selected field's \pm the standard error of the mean. Each clonogenic experiment was repeated twice.

5.3.5: Creation of a mammalian *AKRIC3* expression plasmid and transfection in MCF-7 cells

A plasmid (pENTR211) containing the open reading frame (ORF) for the *AKRIC3* gene was purchased from Invitrogen (Burlington, ON) and transfected into *E. coli* DH5 α cells using standard methods. Successful transformants were cultured on LB-Agar plates containing 50 $\mu\text{g/ml}$ of kanamycin as the selective agent. Plasmid DNA from a positive bacterial clone

(propagated in kanamycin-containing LB medium) was isolated using QIAprep miniprep plasmid extraction kits (Qiagen). The ORF for *AKR1C3* was amplified by PCR using the purified plasmid as template and the following AKR1C3-specific primers (Forward: 5'- GCT AAG ATC TTC ATG GAT TCC AAA CAC CAG TGT G -3', Reverse: 5'- TCG ACT CGA GGT ACA AGA AAG CTG GGT TCT AAT ATT CAT C -3' (IDT)). After cleavage with appropriate restriction endonucleases, the *AKR1C3* cDNA within the amplified ORF product was ligated into identical cleavage sites within the pCMV-FLAG plasmid (Stratagene), such that the FLAG Tag was incorporated in frame with the C-terminal domain of the protein-coding sequence. The ligation reaction was transformed into competent DH5- α *E. coli* bacteria and cultured on kanamycin-containing plates to select for clones harboring the pAKR1C3-FLAG plasmid. The recombinant plasmid was purified from bacterial transformants using Qiagen QIAprep maxiprep kits (Qiagen). Plasmid concentrations and purity were determined by monitoring absorbance at 260 nm and 280 nm. The AKR1C3-Tag insert within pAKR1C3-FLAG was sequenced (MOBIX) to confirm that no mutations were introduced into the AKR sequence during DNA cloning.

Clones of cells stably expressing the AKR1C3 cDNA were generated by transfecting MCF-7_{CC-12} cells with pAKR1C3-FLAG DNA using Lipofectamine 2000TM medium (Invitrogen). Cells were incubated overnight, and then subjected to selective screening in D-MEM medium containing 2.0 mg/ml G418 (Sigma, St. Louis, MO) until G418-resistant clones were obtained. Cells transiently overexpressing *AKR1C3* were also generated by transfection with 24 μ g of plasmid DNA complexed to 60 μ l of Lipofectamine 2000. The cells were transfected for 18 h and then re-plated for subsequent experiments. The amount of

overexpression of AKR1c3 protein was assessed in immunoblotting experiments employing an anti-human AKR1c3 antibody (Sigma, St. Louis, MO) using the protocol described below.

5.3.6: siRNA-mediated knockdown of *AKR1C3* and *AKR1B10* expression

Silencer select siRNA oligoribonucleotides were obtained from Life Technologies (Burlington, ON) to induce the specific degradation of *AKR1C3* and *AKR1B10* transcripts. Two different siRNAs were used to reduce expression of these genes (16448 (KD1) and 225013 (KD2) for *AKR1C3*, and 32583 (KD3) and 32584 (KD4) for *AKR1B10*). In addition, a scrambled control siRNA (SCRAM, also from Life Technologies) was also used as a negative control to identify any possible off-target effects, including those induced by the transfection process itself. Cells were transfected with 5 ng of siRNA using Lipofectamine 2000, as per the manufacturer's directions. Knockdowns were confirmed by quantitative PCR and western blot analyses. The effect of the siRNAs on cellular sensitivity to anthracyclines was also assessed using clonogenic assays.

5.3.7: Immunoblotting experiments with epitope- and phospho-specific antibodies

Cells were lysed by incubation in standard RIPA buffer supplemented with 1x Complete protease inhibitor cocktail (Roche), 10 μ M NaF, 2 mM Na_3VO_4 and 1 μ M PMSF (Sigma, St. Louis, MO). The amount of protein in each whole cell extract was quantified using the BCA assay, as per the manufacturer's protocol (Pierce). Extracts were aliquoted into 1.5 ml tubes containing standard Laemmli sample buffer (1X final concentration) and boiled for 5 min, after which the proteins in the extracts were resolved by standard SDS acrylamide gel electrophoresis procedures. The resolved proteins in the extracts were transferred by electrophoresis to nitrocellulose membranes and the membranes probed with specific primary antibodies and

appropriate HRP-conjugated secondary antibodies using standard immunoblotting procedures. In some instances, blots were stripped for 1 h at 50°C in a buffer containing 6.24 mM Tris pH 6.7, 2% (w/v) SDS, and 100 mM β -mercaptoethanol before re-probing with another antibody.

5.3.8: Assessment of estrogen receptor α and estrogen receptor β transcript expression

The level of human ER α gene (*ESR1*) transcripts in the above cell lines was assessed by quantitative PCR (Q-PCR). Qiagen RNeasy kits (Qiagen) were used to extract total RNA from the above cell lines. Prior to Q-PCR, all RNA samples were assessed for RNA integrity by capillary electrophoresis on an Agilent 2100 Bioanalyzer using RNA 6,000 Nano Assay Lab Chips as per the manufacturer's directions (Agilent Technologies). RNA to be used in Q-PCR experiments had to exhibit an RNA integrity number (RIN) of ≥ 8 and a 28S/18S ratio of ≥ 1.8 . All RNA samples were subjected to DNase treatment prior to reverse transcription as previously described (42). The expression of *ESR1* transcripts was then assessed by Q-PCR as described (42). The *ESR1* primers used were (Forward: 5'-CCACCAACCAGTGCACCATT-3', Reverse: 5'-GGTCTTTTCGATTCCCACCTTTC-3'). All RNA samples were assessed for *ESR1* transcript expression in triplicate with the transcript for the S28 ribosomal protein as the reference gene.

5.3.9: Immunohistochemical Assessment of Estrogen Receptor A Protein Expression

The expression of human ER α protein expression in the above cell lines was assessed by immunohistochemistry with a human ER α antibody. Cells were harvested and the cell pellets, collected after centrifugation, were delivered on ice to the Pathology Department of Health Sciences North, Sudbury, ON. Cell pellets were fixed in a 5% (v/v) formalin solution for 24 h. The samples were then embedded in HistogelTM prior to embedding in paraffin blocks.

Histological slices were taken from the paraffin blocks and prepared for Hematoxylin and Eosin (H&E) staining for visualization of tumor cells and immunohistochemical staining using an HRP-conjugated human ER α antibody.

5.3.10: Quantitative Determination of Cellular Levels of Activated ER α

Levels of active (nuclear) ER α in cells were quantified using human ER α TransAM™ kits purchased from Active Motif (Carlsbad, CA), using the manufacturer's protocol.

5.3.11: Artemisinin-induced Reduction in Cellular ER α Protein Expression

In some experiments, cellular ER α protein expression was strongly reduced by their incubation with Artemisinin (Sigma, St. Louis, MO). This enabled us to assess changes in cellular phenotypes associated with strongly reduced ER α levels. Cells were plated at 30% confluence in 10 cm plates containing D-MEM supplemented with 10% FBS and antibiotics. After cells had adhered for 24 h, the media was removed and replaced with D-MEM supplemented with 10% FBS and either 300 μ M Artemisinin or 0.1% v/v DMSO as the control. Cells were allowed to grow at 37°C at 5% CO₂ for 72 h. Following the treatment period, proteins were extracted from the cells as previously described.

5.3.12: Data Analysis

All graphs were prepared using GraphPad Prism V5.0 software. All data points depicted represent the mean \pm standard error of mean (SEM). Statistical analyses were performed using GraphPad Prism V 5.0 software. Analysis of Variance (ANOVA) tests were performed, assuming a normal distribution for all data sets, followed by a Bonferoni *post hoc* test for significance. Comparisons were made between drug-selected and co-cultured control cell lines at various selection doses, as well as between treated and untreated cell lines or between cells

expressing and not expressing specific transcripts or siRNAs. Due to the multi-parametric nature of the data analyses, an ANOVA was chosen as the most suitable method of data analysis. Differences between samples or cell lines were deemed to be significant if they had a p value of < 0.05 for the above statistical tests.

5.4: Results

5.4.1: Expression of AKR1C Isoforms

Previously conducted DNA microarray studies comparing gene expression between MCF-7_{CC-12} and MCF-7_{DOX2-12} cells identified a number of differences in gene expression that accompany the acquisition of doxorubicin resistance (13). Among these were members of the AKR 1C gene family, although the probes used in the microarray experiments could not effectively distinguish between the various transcripts of AKR 1C isoforms. Subsequent Q-PCR experiments using isoform-specific primers confirmed that both *AKR1C2* and *AKR1C3* transcripts are elevated in MCF-7_{EPI-12} and MCF-7_{DOX2-12} cell lines (compared to MCF-7_{CC-12} cells). *AKR1C3* transcript levels were 11.9 ± 3.9 -fold higher in MCF-7_{DOX2-12} cells line ($p < 0.0001$) and 4.5 ± 0.9 -fold higher in MCF-7_{EPI-12} cells compared to the MCF-7_{CC-12} cells. Similarly, *AKR1C2* transcript levels were 5.8 ± 1.1 -fold and 4.6 ± 1.2 -fold higher in MCF-7_{EPI-12} and MCF-7_{DOX2-12} cells compared to MCF-7_{CC-12} cells, respectively (13).

Given that *AKR1C3* was the most strongly upregulated aldo-keto reductase gene in the anthracycline-resistant cell lines, we recently assessed protein extracts of the above cell lines across the various selection doses for their level of AKR1c3 protein expression, as measured using standard immunoblotting procedures (**Figure 5-1 A**). As shown in **Figure 5-1 B**, no statistically significant differences in AKR1c3 expression were observed between MCF-7_{DOX2-}

₇ or MCF-7_{DOX2-8} cells and their co-cultured control cell lines (MCF-7_{CC-7} and MCF-7_{CC-8} cells). However, beginning at selection dose 9 (MCF-7_{DOX2-9}), we observed an 8.7 ± 1.5 fold ($p \leq 0.01$) induction in AKR1c3 protein expression relative to its co-cultured control cell line. MCF-7_{DOX2-10} cells showed a 7.4 ± 1.4 fold ($p \leq 0.05$) induction, while cells selected to doses 11 and 12 showed 12.5 ± 2.3 -fold and 10.2 ± 4.5 -fold inductions, ($p \leq 0.001$ and $p \leq 0.01$, respectively; **Figure 5-1 B**). Interestingly, the expression of another AKR isoform not involved in estrogen biosynthesis (AKR1b10) also increased in a dose-dependent manner as the doxorubicin selection dose was increased beyond selection dose 8 (**Figure 5-1 A**). In contrast, continued propagation of MCF-7 cells in the absence of doxorubicin (MCF-7_{CC} cells) resulted in reduced AKR1c3 protein expression, suggesting that cell propagation under optimal cellular conditions in the absence of a cell stressor (such as doxorubicin) reduces the expression of aldo-keto reductases.

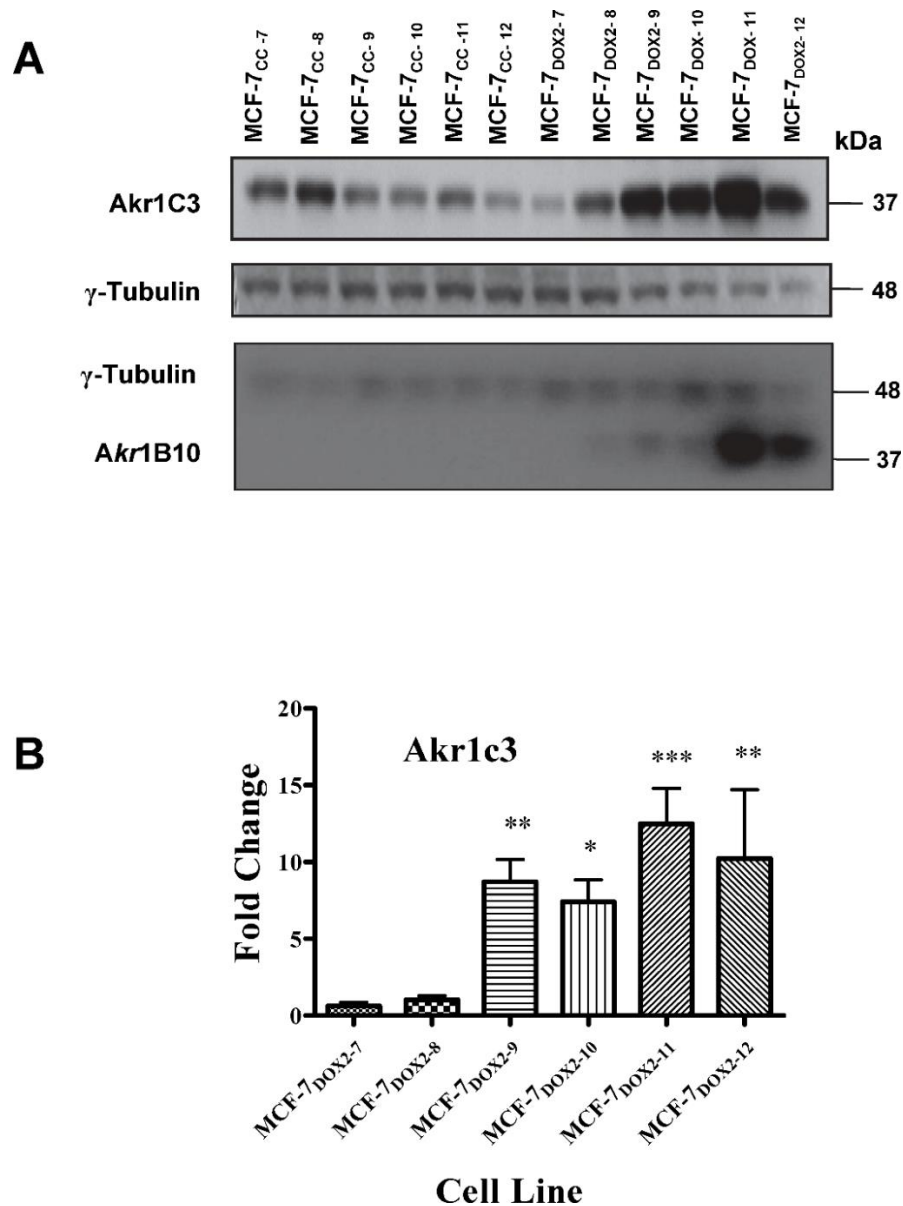


Figure 5-1 Assessment of *AKR1c3* and *AKR1b10* and γ -tubulin expression in various stable cell lines using immunoblotting approaches.

(A) Representative immunoblots for assessing *AKR1c3*, *AKR1b10* and γ -tubulin protein expression in extracts of unselected MCF-7_{CC} cells or doxorubicin-selected MCF-7_{DOX2} cells (selection doses 7 through 12). Blots represent one of 4 independent experiments. (B) Fold change in *AKR1c3* levels relative to corresponding co-cultured control cell lines for doxorubicin-selected cell lines across selection doses 7 through 12 (based by densitometry). Fold changes are expressed as the average \pm S.E.M. for 4 independent experiments. The significance of differences in *AKR1c3* expression between the designated doxorubicin-elected cell line and its corresponding co-cultured control cell line was assessed using an ANOVA test, followed by a Bonferroni correction. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

5.4.2: Transient and Stable Overexpression of AKR1c3 Protein in MCF-7_{CC-12} Cells

We then assessed whether stable or transient expression of recombinant *AKR1C3* transcripts in MCF-7_{CC-12} cells could, by itself, induce doxorubicin resistance. **Figure 5-2** depicts the level of AKR1c3 protein expression in untreated MCF-7_{CC-12} cells, MCF-7_{CC-12} cells exposed to transfection conditions with DNA empty vector control, and MCF-7_{CC-12} cells transiently (panel A) and stably (panel B) transfected with a human *AKR1C3* expression vector (pCMV-AKR1C3-FLAG). Cellular levels of AKR1c3 protein were determined in immunoblotting experiments using an AKR1c3-specific antibody, with a γ -tubulin antibody being used as a loading control. After densitometric quantification of the AKR1c3 bands, the levels of total AKR1c3 expression relative to MCF-7_{CC-12} cells was then determined for the transiently and stably transfected cells. We found that while transient transfections did not result in permanent elevations in AKR1c3 expression, the fold increase in total AKR1c3 immunoreactivity was much greater (up to 5-fold) for transiently transfected MCF-7_{CC-12} cells than stable transformants (up to 1.7-fold). It should be noted that during optimization experiments, AKR1c3 levels were found to increase when cells were treated with an empty vector (pCMV-FLAG; see **Figure 5-2 A**), suggesting that the stresses associated with the transfection process are sufficient to induce AKR1c3 expression. This was confirmed in subsequent experiments where cells transfected under identical conditions in the absence of expression plasmids also exhibited increased AKR1c3 expression (data not shown).

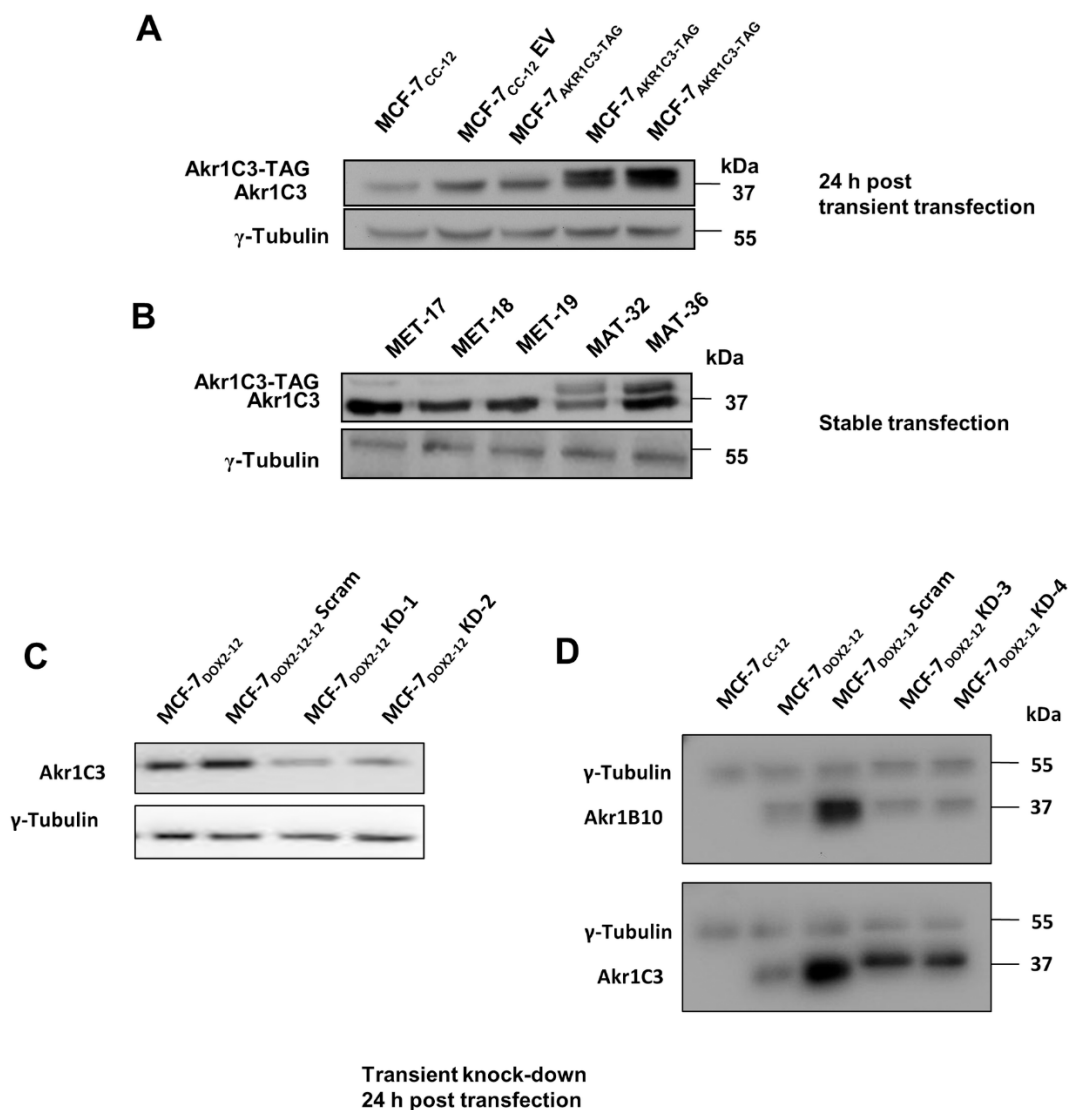


Figure 5-2 Assessment of AKR1c3, AKR1b10 and γ -tubulin expression in various stably and transiently transfected cell lines using immunoblotting approaches.

A) Optimization of transfection using lipofectamine 2000. Lane 1 represents untreated MCF-7_{CC-12} cells, lane 2 is for identical cells transfected with an empty vector (pCMV-FLAG) in the presence of lipofectamine (1:2 ratio), while lanes 3, 4 and 5 are cells transfected with FLAG-tagged AKR1c3 expression vector (pCMV-AKR1C3-FLAG) at vector to lipofectamine ratios of 1:1, 1:1.5 and 1:2, respectively. B) A representative western blot assessing AKR1C3 protein expression in MCF-7_{CC-12} cells stably transfected with an empty vector (lanes 1, 2 and 3) or pAKR1C3-FLAG (lanes 4 and 5). C) A representative western blot assessing AKR1c3 protein expression in cells transfected with a random RNA sequence (scrambled) or with an AKR1C3-specific siRNAs (KD-1 and KD-2). D) A representative western blot assessing AKR1b10 and AKR1c3 protein expression in cells transfected with a scrambled siRNA or AKR1B10-specific siRNAs (KD-3 and KD-4).

5.4.3: Effect of AKR1c3 Overexpression on Cellular Doxorubicin Sensitivity

We then assessed whether the transient or stable expression of recombinant AKR1c3 alters the sensitivity of MCF-7_{CC-12} cells to doxorubicin using a clonogenic assay. The two clones having the highest stable expression of recombinant AKR1c3 (MAT36 and MAT32, **Figure 5-2 A**) had a higher IC₅₀ for doxorubicin (110 ± 93 nM) than stable clones with little to no AKR1C3 expression (46 ± 29 nM) (see **Figure 5-3 A**). This translates into a 2.4-fold decrease in doxorubicin sensitivity, consistent with a possible role for AKR1c3 in doxorubicin resistance; however, this difference was not deemed to be significant using an ANOVA. Perhaps the modest fold increase (1.5-fold) in total AKR1C3 expression was insufficient to confer a statistically significant reduction in doxorubicin sensitivity. We thus examined whether the ~5-fold change in total AKR1C3 expression found in MCF-7_{CC-12} cells transiently transfected with pCMV-AKR1C3-FLAG might be sufficient to induce statistically significant changes in doxorubicin sensitivity. As shown in **Figure 5-3 B**, no significant change in doxorubicin sensitivity was observed. The IC₅₀ for doxorubicin in untransfected MCF-7_{CC-12} cells was 16.2 nM, while mock-transfected and pCMV-AKR1C3-FLAG-transfected cells had IC₅₀s for doxorubicin of 14.4 nM and 12.7 nM, respectively. Taken together, the data suggests that simply increasing the expression of one AKR isoform (AKR1c3) was insufficient to confer doxorubicin resistance in MCF-7_{CC-12} cells.

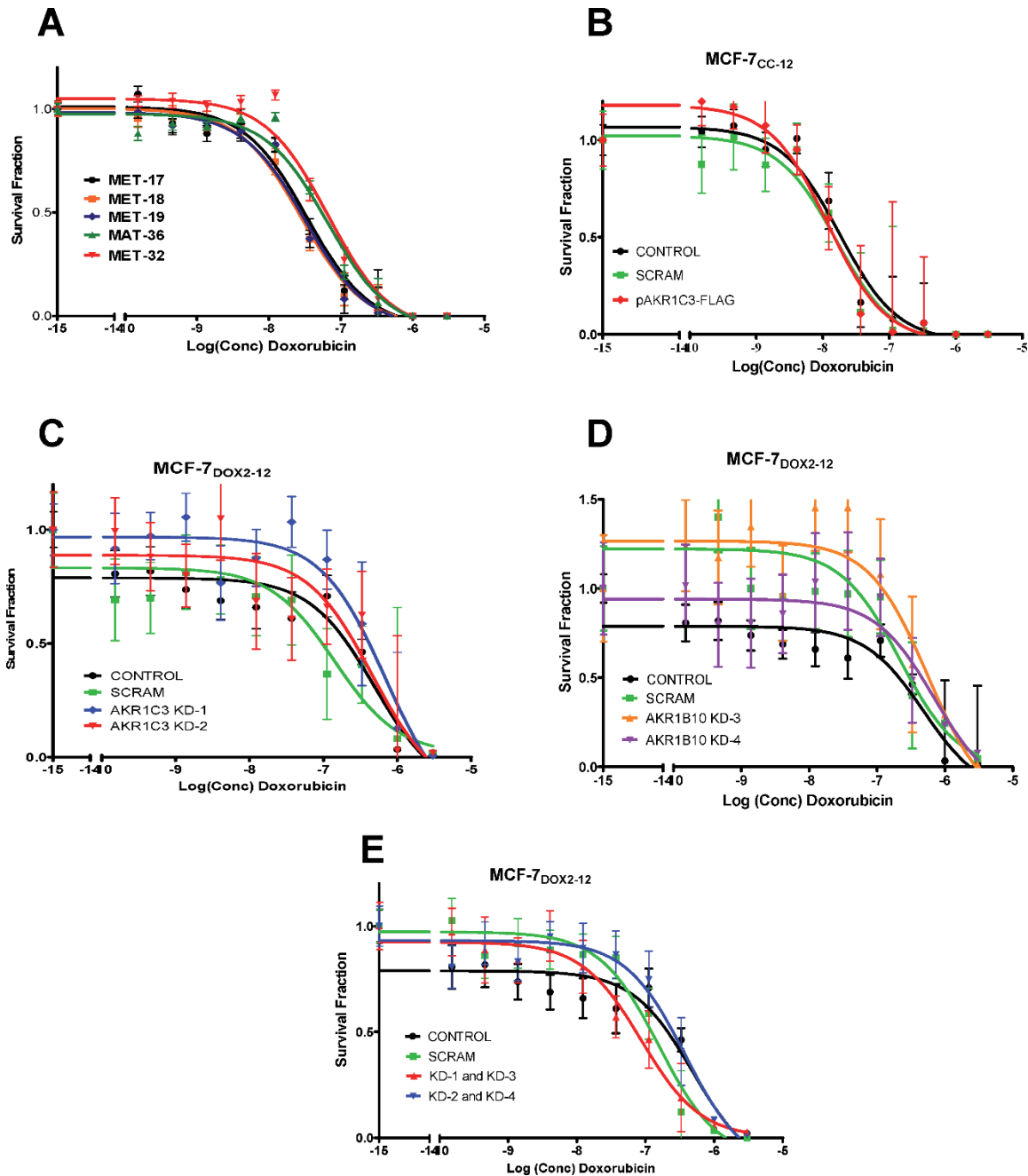


Figure 5-3 Differences in sensitivity to doxorubicin, as measured using clonogenic assays for MCF-7_{CC-12} cells stably or transiently transfected with various constructs.

Survival curves are depicted for: (A) stable clones of MCF-7_{CC-12} cells transfected with pAKR1C3-FLAG, (B) MCF-7_{CC-12} cells, with or without transient transfection with the pAKR1C3-FLAG plasmid, (C) MCF-7_{DOX2-12} cells, with or without transient transfection with scrambled or AKR1C3-specific siRNAs, (D) MCF-7_{DOX2-12} cells, with or without transient transfection with scrambled or AKR1B10-specific siRNAs, (E) MCF-7_{DOX2-12} cells, with or without transient transfection with scrambled or both AKR1C3- and AKR1B10-specific siRNAs.

In order to further assess the relationship between the expression of AKRs and doxorubicin resistance, we then assessed the effect of *AKR1C3*- and *AKR1B10*-specific siRNAs on doxorubicin sensitivity in MCF-7_{DOX2-12} cells. siRNAs against *AKR1C3* were designated KD1 and KD2, while siRNAs against *AKR1B10* were designated KD3 and KD4. Interestingly, transfection of MCF-7_{DOX2-12} cells with a scrambled control siRNA strongly increased both AKR1c3 and AKR1b10 expression (**Figure 5-2 D**), similar to our observations of increased AKR1c3 expression in MCF-7_{CC-12} cells upon transfection with an empty vector (pCMV-FLAG; **Figure 5-2 A**). Nevertheless, transient transfection of MCF-7_{DOX2-12} cells with the *AKR1C3*- or *AKR1B10*-specific siRNAs resulted in an average reduction in AKR1c3 or AKR1b10 expression of 50 to 80% relative to the scrambled control siRNA (**Figure 5-2 C and D**, respectively). The KD-1 and KD-3 siRNAs gave the highest knockdown of AKR1c3 and AKR1b10 protein expression, respectively (at near 80%; **Figure 5-2 C and D**). Despite the high data variability, the IC₅₀s for doxorubicin in MCF-7_{DOX2-12} cells transfected with the *AKR1C3* siRNAs (680 nM and 460 nM), were very similar to that of untransfected MCF-7_{DOX2-12} cells (490 nM; see **Figure 5-3 C**). Similarly, knockdown of AKR1b10 expression in MCF-7_{DOX2-12} cells resulted in IC₅₀s for doxorubicin of 560 nM and 690 nM for the two siRNAs (**Figure 5-3 D**), very similar to that of MCF-7_{DOX2-12} cells. Knockdown of one of the AKR isoforms did not induce an upregulation of the expression of the other AKR isoform (**Figure 5-2 D**). To address possible effects of compensatory gains in the expression of one AKR isoform due to the loss of another isoform, we performed a dual-knockdown experiment in which we treated cells with *both* *AKR1C3*- and *AKR1B10*-specific siRNAs (KD1 and KD3 or KD2 and KD4).

Transfection of MCF-7_{DOX2-12} cells with both siRNAs did not result in any significant change in doxorubicin sensitivity (**Figure 5-3 E**).

5.4.4: Factors Affecting E1 to E2 Conversion in Various Breast Tumor Cell Lines

Since AKR1C3 is known to play a role in E1 to E2 conversion, the above-documented increases in AKR1C3 expression in MCF-7_{DOX2} cells and MCF-7_{EPI} cells would be expected to result in higher cellular E2 levels. Given that E2 promotes cell growth and survival in breast tumor cells (43, 44), the elevated E2 levels could, in turn, contribute to the observed anthracycline resistance phenotype. To assess the former hypothesis, MCF-7_{CC-12}, MCF-7_{DOX2-12}, and MCF-7_{EPI-12} cells (selected to dose level 12) were treated with exogenous E1 (100 nM) and the amount of E2 released in the medium determined in the presence or absence of various pathway inhibitors. These included letrozole (which inhibits the aromatization of androgens androstenedione and testosterone to estrone and estradiol, respectively) and β -cholanolic acid, a known AKR inhibitor. E2 secreted into the media of cells was monitored over a 24 h period. By inhibiting the aromatase pathway we were able to monitor the effects of β -cholanolic acid on the direct conversion of E1 to E2. As shown in **Figure 5-4 A**, even in the absence of exogenously added E1, both MCF-7_{DOX2-12} and MCF-7_{EPI-12} cells secreted elevated levels of E2 in the medium relative to MCF-7_{CC-12} cells. This is consistent with the induction of AKR1c3 and the enzyme's ability to promote the conversion of endogenous E1 to E2. Upon addition of exogenous E1, media levels of E2 dramatically increased in all examined cell lines (MCF-7_{CC-12}, MCF-7_{EPI-12} and MCF-7_{DOX2-12} cells). Pre-treatment of cells with the aromatase inhibitor letrozole (100 ng/ml) for 1 h prior to the addition of 0.1 μ M exogenous E1 reduced E2 secretion into media, but this reduction was only significant for MCF-7_{EPI-12} cells ($p \leq 0.001$). Pre-treatment of cells with the AKR inhibitor β -cholanolic acid (200 μ M) for 1 h prior to the addition of E1 had little effect on

media E2 levels for MCF-7_{CC-12} cells (1.8±0.2 ng/ml), consistent with the considerably lower levels of AKR1C3 expression in MCF-7_{CC-12} cells compared to MCF-7_{EPI-12} and MCF-7_{DOX2-12} cells. In contrast, the latter two cell lines exhibited significant reductions in media E2 levels in the presence of β -cholanolic acid ($p \leq 0.01$ and $p \leq 0.001$, respectively).

Stable AKR1c3-FLAG-expressing clones (MET-17, MAT-32, and MAT-36) were analyzed for E2 levels in the absence or presence of E1 and/or pharmacological inhibitors of aromatase or AKR1c3. While all stable transfectants exhibited dramatically enhanced E2 production in the presence of exogenous E1, there were no differences in E2 production among the E1-treated stable transfectants. The addition of letrozole had no effect on E1 to E2 conversion, while β -cholanolic acid treatment consistently reduced E1 to E2 conversion in stably transfected cells, but these reductions were not statistically significant using an ANOVA test (**Figure 5-4 B**). Treatment with E1 also induced strong E1 to E2 conversion in transiently transfected cells, and even in untransfected and mock-transfected cells (**Figure 5-4 C**). Similar to the stably transfected cells, addition of letrozole had no effect on the conversion of E1 to E2. In contrast, β -cholanolic acid treatment significantly reduced E1 to E2 conversion in mock ($p \leq 0.01$) and pAKR1C3-FLAG-transfected cells ($p \leq 0.05$). Untransfected wildtype cells also exhibited a reduction in E2 production, but this was not significant using an ANOVA test (**Figure 5-4 C**). As only AKR1C3 is known to convert E1 to E2 conversion, AKR1B10 overexpressing constructs were not assessed for their effects on E2 metabolism.

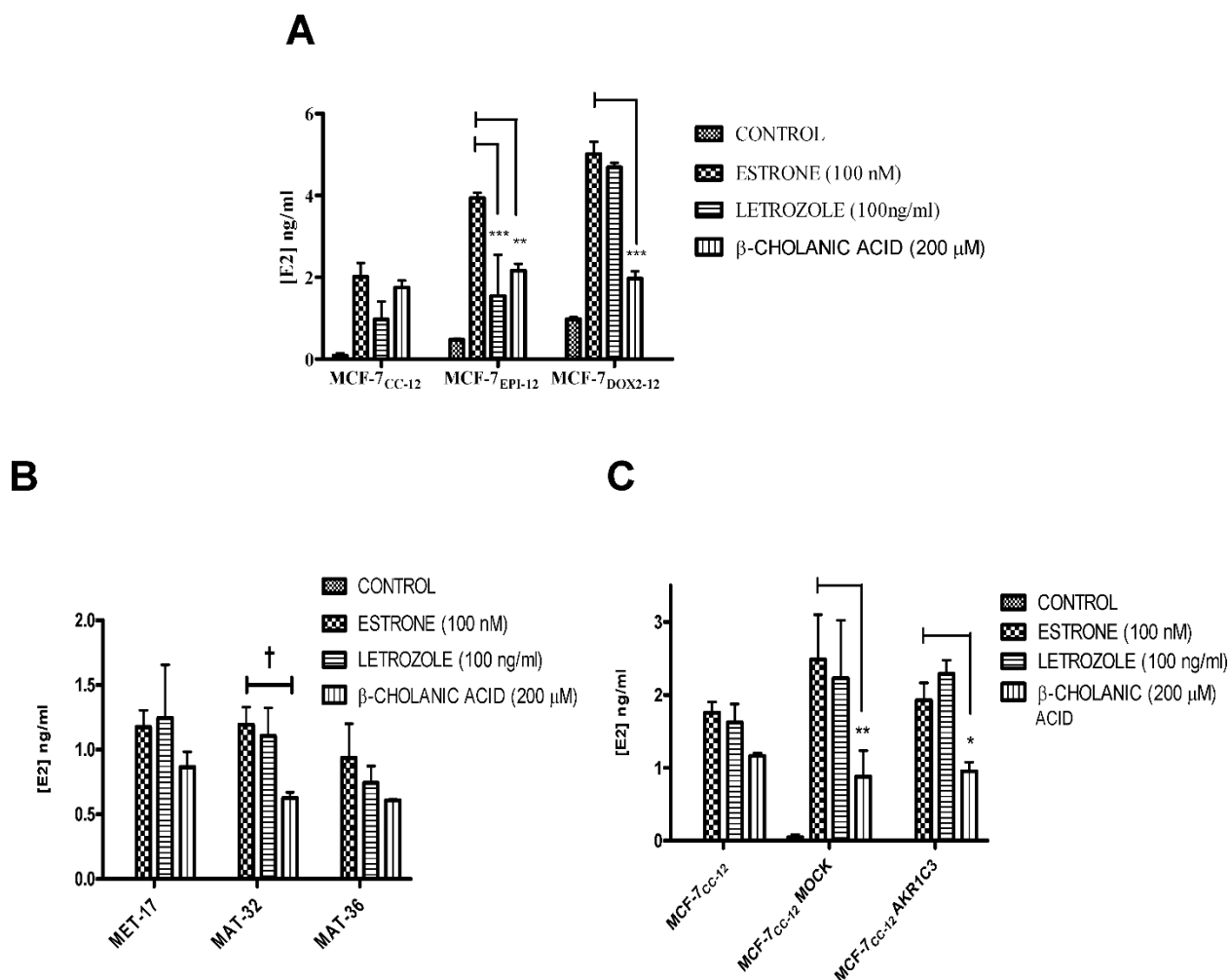


Figure 5-4 Degree of Production of Estradiol (E2) from Estrone (E1) in various cell lines under varying conditions.

(A) MCF-7_{DOX2-12}, MCF-7_{EPI-12}, and MCF-7_{CC-12} cells. (B) Clones of MCF-7_{CC-12} cells stably transfected with pAKR1C3-FLAG. (C) MCF-7_{CC-12} cells, with or without transient transfection with pAKR1C3-FLAG. Some cells were pre-treated with an AKR inhibitor (β-cholanic acid) or an aromatase inhibitor (letrozole) for 1 h prior to the addition of 100 nM estrone. Estradiol levels were quantified using E2 ELISA kits. Bars represent the mean from 3 independent trials with technical duplicates ± S.E.M. Statistical analysis was done using an ANOVA test, followed by a Bonferroni correction. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.4.5: Changes in ERα Transcript Levels upon Selection for Anthracycline resistance

The elevated E2 production in anthracycline-resistant cell lines would be expected to result in their enhanced proliferation (20, 25, 27) through E2's ability to bind to and activate ERα

(45, 46). This is providing ER α expression and activity is unaltered upon selection for anthracycline resistance. We thus examined the level of expression of ER α transcripts throughout selection of MCF-7 cells for survival in increasing concentrations (doses) of doxorubicin or epirubicin. As shown in **Figure 5-5 A**, during selection for doxorubicin resistance, dose 7 cells exhibited a 2.0 ± 0.2 -fold ($p \leq 0.05$) reduction in ER α transcript (*ESR1*) levels compared to MCF-7_{CC-12} cells. As selection progressed through doses 8 through 12, *ESR1* transcript levels further decreased by up to 6-fold ($p \leq 0.01$ for doses 8–12). MCF-7 cells selected for resistance to epirubicin (MCF-7_{EPI-12} cells) also showed a 2.6 ± 0.1 -fold ($p \leq 0.01$) reduction in *ESR1* levels relative to MCF-7_{CC-12} cells (**Figure 5-5 A**). All Q-PCR Ct values were normalized to the expression of the S28 reference gene. All fold changes in transcript levels were expressed relative to levels in MCF-7_{CC-12} cells. No significant differences in ER α transcript levels were observed upon selection in the absence of doxorubicin or epirubicin (MCF-7_{CC} selection doses 7 through 12; **Figure 5-5 B**).

5.4.6: Estrogen Receptor Expression and Activity Levels in Anthracycline-resistant Cells

We then conducted immunoblotting experiments to assess changes in ER α protein levels upon selection for anthracycline resistance (**Figure 5-6 A-C**). Consistent with *ESR1* transcript levels, we did observe reductions in ER α protein expression in both MCF-7_{EPI-12} and MCF-7_{DOX2-12} cells relative to MCF-7_{CC-12} cells, although only the decrease in MCF-7_{DOX2-12} cells was found to be statistically significant using an ANOVA test ($p < 0.001$). Interestingly, as shown in **Supplemental Figure 5-1**, no changes in the expression of estrogen receptor β (ER- β) were observed during selection for doxorubicin resistance (selection doses 7 through 12).

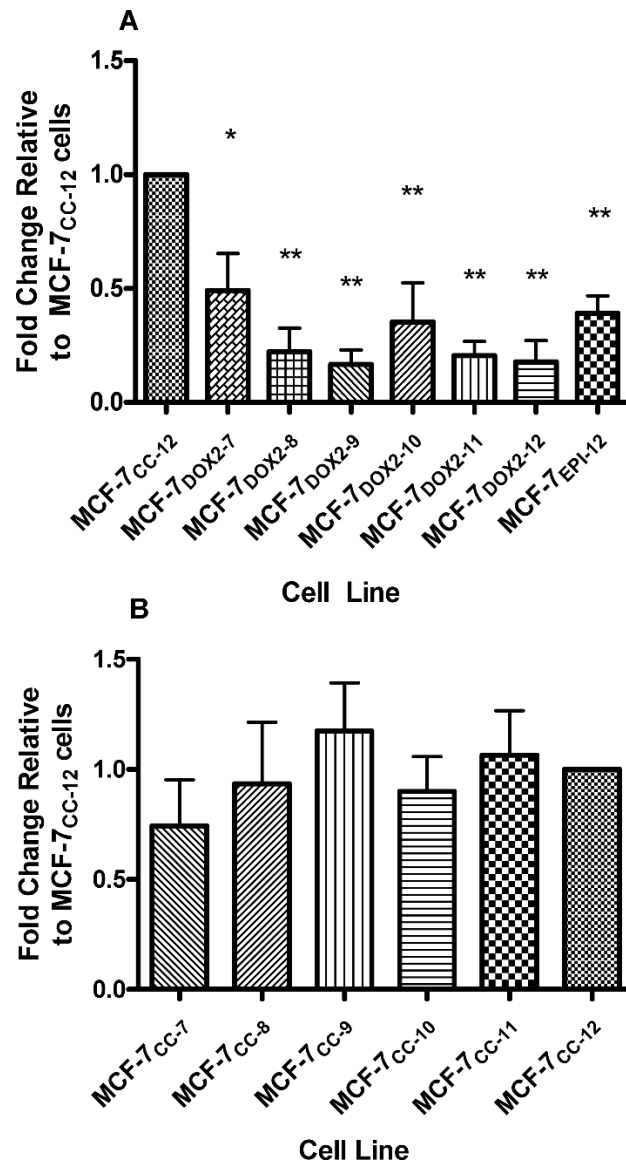


Figure 5-5 Differences in *ESR1* transcript levels in various cell lines, as determined by quantitative PCR (Q-PCR).

(A) *ESR1* transcript levels in MCF-7 cells selected for survival in increasing concentrations (doses) of doxorubicin or epirubicin (relative to MCF-7_{CC-12} cells). Data for doxorubicin selection doses 7 through 12 and for epirubicin at selection dose 12 are depicted. (B) A similar approach was used to quantify *ESR1* transcript levels in MCF-7 cells selected in the absence of drug to similar passage numbers as MCF-7_{DOX2} cells (selected to dose levels 7 through 12). All *ESR1* expression values were normalized to the expression of transcripts for ribosomal protein S28. Values depicted represent the mean \pm S.E.M. for three independent trials. The significance of differences in *ESR1* transcript levels between the designated cell line and MCF-7_{CC-12} cells was assessed using an ANOVA test, followed by Bonferoni correction. $n = 3$, * $p < 0.05$, ** $p < 0.001$

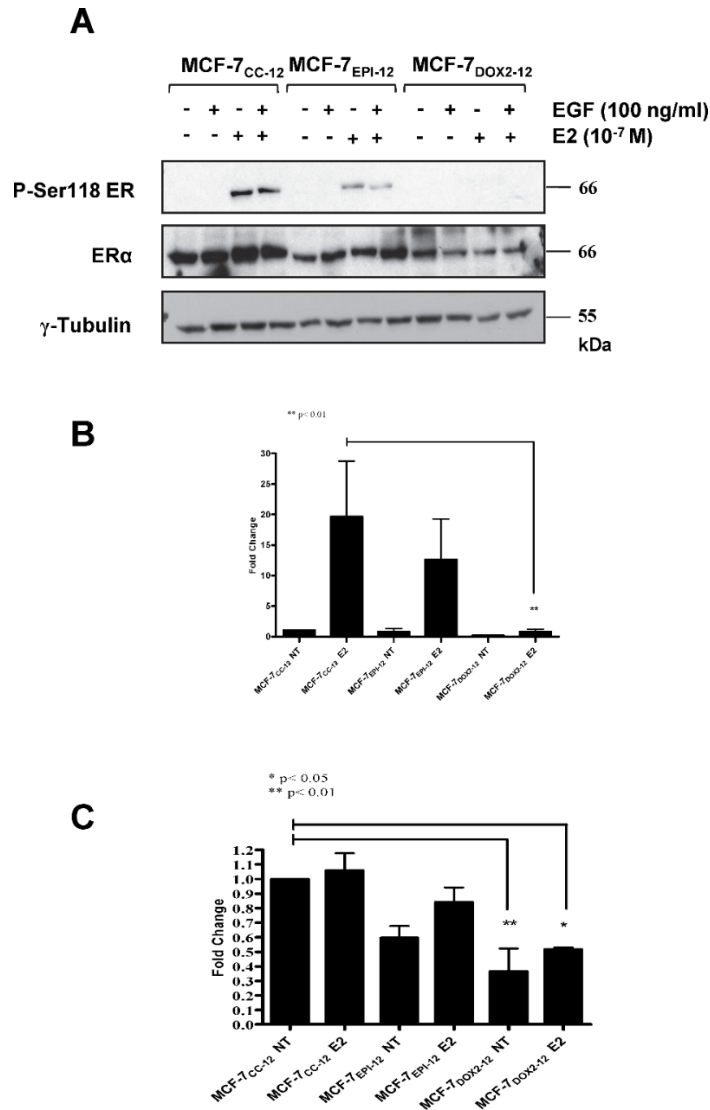


Figure 5-6 Differences in the expression of estrogen receptor α (ER α), phosphorylated estrogen receptor α at serine 118 (P-Ser118 ER), and γ tubulin in various cell lines, as measured in immunoblotting experiments with epitope- or phospho-specific antibodies.

(A) Immunoblots were conducted using extracts of cells with or without incubation in the presence of 100 nM E2, 100 mg/ml epidermal growth factor (EGF), or a combination of E2 and EGF. Primary antibodies purchased from Cell Signaling and used at 1:1,000 dilution in 0.5% BSA overnight at 4°C. (B) Fold change in Ser-118 phosphorylated estrogen receptor relative to untreated MCF-7_{CC-12} cells, normalized to γ -tubulin expression (left) and to basal ER α expression (right). Data is expressed as the mean fold change observed in 3 independent experiments (\pm S.E.M., with the value of untreated MCF-7_{CC-12} cells set to 1.0. (C) Fold change in ER α levels, in a manner identical to that of P-Ser118 ER. The significance of differences between the test sample and that of untreated MCF-7_{CC-12} cells was assessed using an ANOVA test, followed by Bonferoni correction. * = $p < 0.05$, ** = $p < 0.001$

The observation that E1 to E2 conversion was increased in MCF-7_{EPI-12} and MCF-7_{DOX2-12} cells, prompted an analysis of whether this was associated with increased levels of phosphorylated (active) ER α , as determined using an antibody that binds to ER α when phosphorylated at Ser118. Cells were serum-starved overnight and then treated in hormone-depleted media for 30 min with either 100 nM E2, 100 ng/ml EGF, or a combination of the two agents. Previously published studies have shown that E2 and EGF are strong activators of ER α phosphorylation (29, 31, 44). As shown in **Figure 5-6 A**, E2 (but not EGF) was able to strongly induce ER α phosphorylation in MCF-7_{CC-12} cells. Interestingly, ER α phosphorylation was undetectable and considerably lower in MCF-7_{DOX2-12} and MCF-7_{EPI-12} cells, respectively, relative to that seen in MCF-7_{CC-12} cells (**Figure 5-6 A**). Only levels of phosphorylated ER α in MCF-7_{DOX2-12} cells were found to be significantly reduced relative to MCF-7_{CC-12} cells in the presence of exogenous E2 ($p \leq 0.01$). This finding held true even when levels of phosphorylated estrogen receptor were standardized to total estrogen receptor (**Figure 5-6 B**). In three independent experiments, treatment of MCF-7_{CC-12} cells with E2, EGF or a combination of the two had little to no effect on ER α receptor levels. However, both MCF-7_{EPI-12} and MCF-7_{DOX2-12} cells showed overall reductions in ER α receptor levels.

In subsequent experiments (**Figure 5-7**), we examined changes in ER α receptor levels during selection for doxorubicin resistance (selection doses 7 to 11). Cells exhibited reductions in both phosphorylated ER α and total ER α levels during selection for doxorubicin resistance, particularly the former. Reductions in total ER α expression relative to untreated MCF-7_{CC-10} were first observed as early as selection dose 7 relative to untreated co-culture control cells. This was mirrored by a similar reduction in ER α levels in cells treated with E2. The total ER α levels continued to decline at higher selection doses (**Figure 5-7**). Reductions in P-Ser118-ER α levels

across the selection doses mirrored that observed above for ER α levels with significant reductions observed at doses 8 and 9 ($p \leq 0.001$) and dose 10 ($p \leq 0.01$), when compared to MCF-7_{CC-10} cells treated with E2.

5.4.7: Changes in ER α Transcriptional Activity in Anthracycline Resistance

The observed reductions in ER α expression and phosphorylation (activation) upon selection for doxorubicin resistance would be expected to result in reduced ER α activity, as assessed by the receptor's ability to bind the E2 response element (ERE). For this assessment a TransAMTM kit from Active Motif, Inc. (Carlsbad, CA) was used, where the amount of ER α binding to the ERE sequence in the presence of E2 was expressed as the corrected absorbance at 450 nm (after subtraction of background) divided by the corrected absorbance at 655 nm (after subtraction of background). As shown in **Figure 5-8**, MCF-7_{CC} and MCF-7_{DOX2} cells at selection dose 7 had very similar absorbance ratios. However, at selection dose 12 (where significant doxorubicin resistance is obtained), the absorbance ratio for MCF-7_{CC} cells increased, while the absorbance for MCF-7_{DOX2-12} cells decreased. This represented roughly a significant 2.6-fold reduction in the level of active ER α in the nucleus of MCF-7_{DOX2-12} cells ($p \leq 0.01$).

The reduction in the ability of ER α to bind the ERE sequence in MCF-7_{DOX2} cells would be expected to result in a concomitant reduction in the expression of genes whose expression is positively regulated by ER α namely Bcl2 and cyclin D1 (45, 47, 48). We thus examined in immunoblotting experiments the expression of BCL-2 and Cyclin D1 in MCF-7_{CC} and MCF-7_{DOX2} cells at selection doses 7 and 12 using antibodies that specifically recognize these proteins (**Figure 5-9 A and C**, respectively). Expression of these proteins was quantified by densitometry

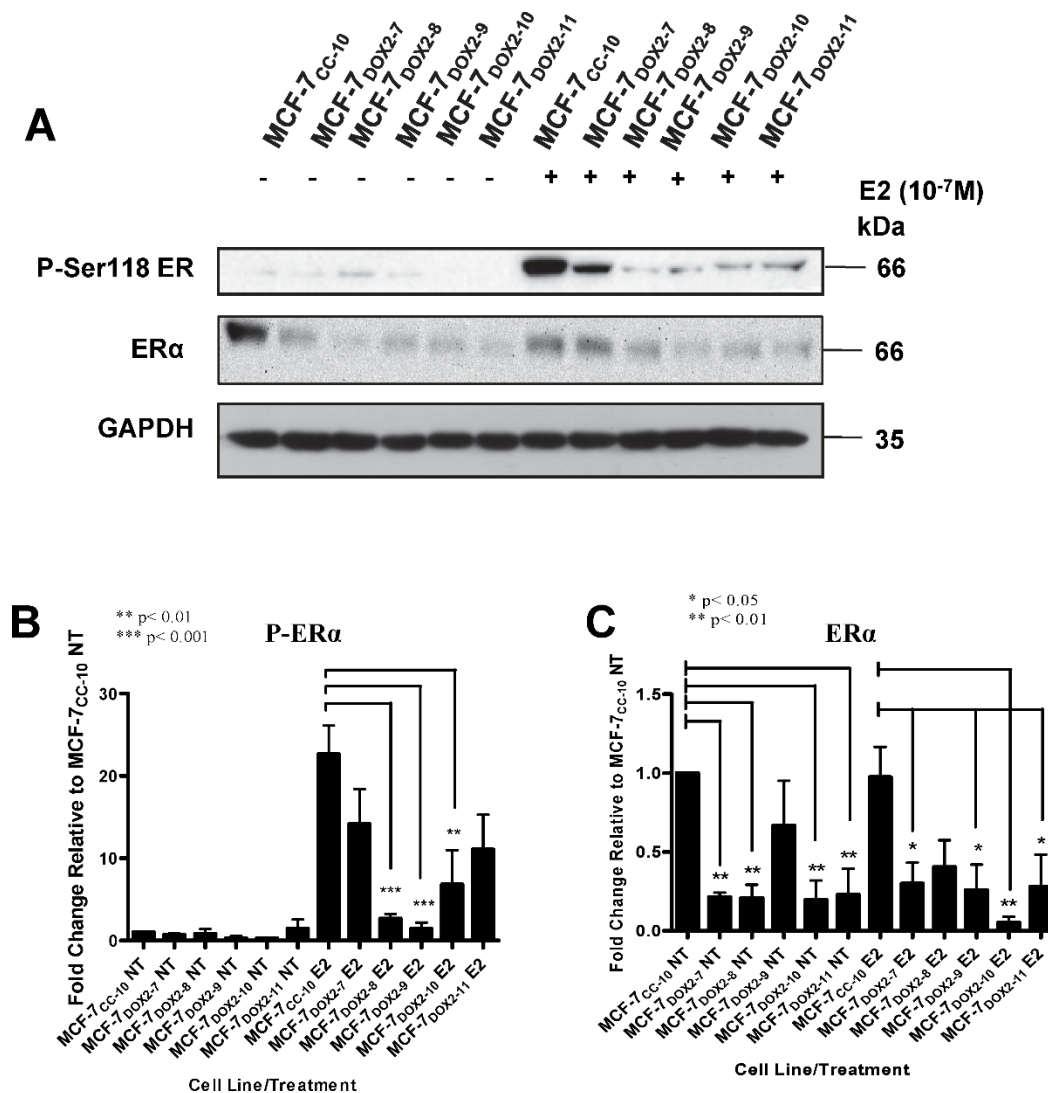


Figure 5-7 Differences in the expression of estrogen receptor α (ER α), phosphorylated estrogen receptor α at serine 118 (P-Ser118 ER), and GAPDH during selection for survival in the absence or presence of increasing concentrations (doses) of doxorubicin

Immunoblots were conducted using extracts of cells without or with a treatment with 100 nM E2. Primary antibodies purchased from Cell Signaling and used at 1:1,000 dilution in 0.5% BSA overnight at 4°C. (A) Representative western blots for Era and GAPDH. (B) Data for P-Ser118 ER is expressed relative to untreated (NT) MCF-7_{CC-12} cells. (C) Data for ER α is expressed relative to untreated (NT) MCF-7_{CC-12} cells. All expression values were first normalized to GAPDH expression. Bars represent the mean \pm S.E.M. for three independent experiments. The significance of differences between the test sample and that of treated or untreated MCF-7_{CC-12} cells was assessed using an ANOVA test, followed by Bonferoni correction. * p < 0.05, ** p < 0.01, *** p < 0.001

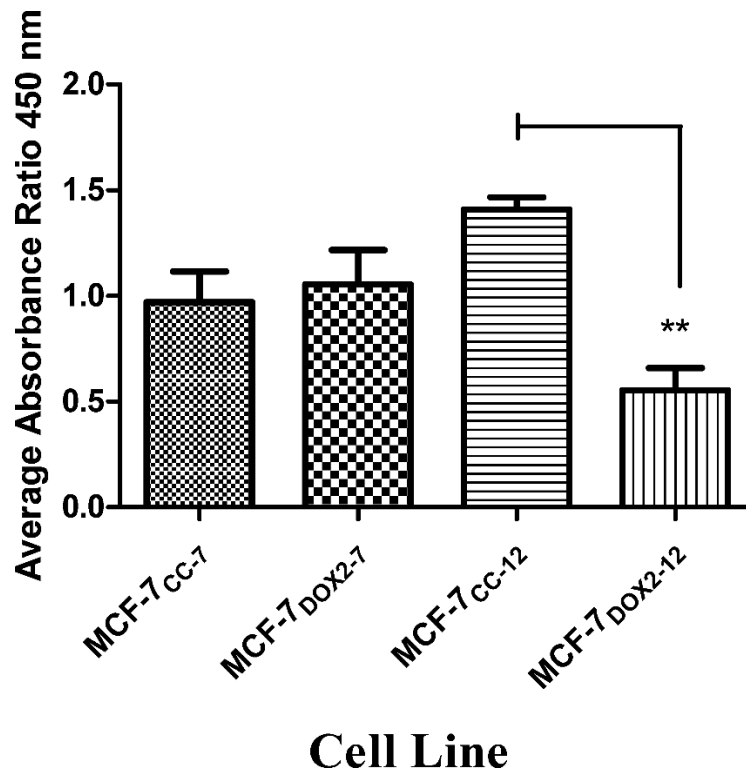


Figure 5-8 Relative levels of active ER α in nuclear extracts.

Active ER α levels were measured in MCF-7_{CC} cells and MCF-7_{DOX2} cells at selection doses 7 and 12, as determined by ER α TRANS-AM kits. Absorbance were corrected for background and expressed as a ratio (450 nm to 655 nm). Extracts were performed under basal conditions with cells grown in D-MEM to 90% confluence. Values depicted are the mean \pm S.E.M. of three independent experiments, each made up of a duplicate technical replicate. The significance of differences in active ER α levels between samples was assessed using an ANOVA test, followed by Bonferoni testing. n = 3, ** p< 0.01

and expressed relative to that of a reference protein (γ tubulin). As shown in **Figure 5-9 A and B**, BCL-2 levels were significantly higher ($p \leq 0.001$) in MCF-7_{DOX2} cells at selection dose 7 compared to MCF-7_{CC} cells at similar passage number (MCF-7_{CC} cells at dose 7). The elevated expression of this apoptosis inhibitor may have helped facilitate resistance to doxorubicin at the early selection doses. However, BCL-2 levels were dramatically reduced in MCF-7_{DOX2} cells as selection progressed to dose level 12 compared to MCF-7_{CC-12} cells ($p \leq 0.001$). This would be

consistent with the reduced and dramatically reduced levels of ER α and phosphorylated ER α in MCF-7_{DOX2-12} cells relative to MCF-7_{CC-12} cells, respectively (**Figure 5-6**). Cyclin D1 levels were slightly reduced upon selection for doxorubicin resistance to dose level 7 (MCF-7_{DOX2-7} cells) compared to MCF-7_{CC-7} cells. At selection dose 12, however, the difference in cyclin D1 expression between MCF-7_{DOX2-12} and MCF-7_{CC-12} cells became significantly different ($p \leq 0.001$) and could be due to reduced levels of ER α and phosphorylated ER α in MCF-7_{DOX2-12} cells. Interesting, the expression of a number of ER- β -dependent genes (CCNA1, HSD11B2, and TMOD1), as measured by quantitative PCR, was not significantly changes during selection for doxorubicin resistance (**Supplemental Figure 5-1**).

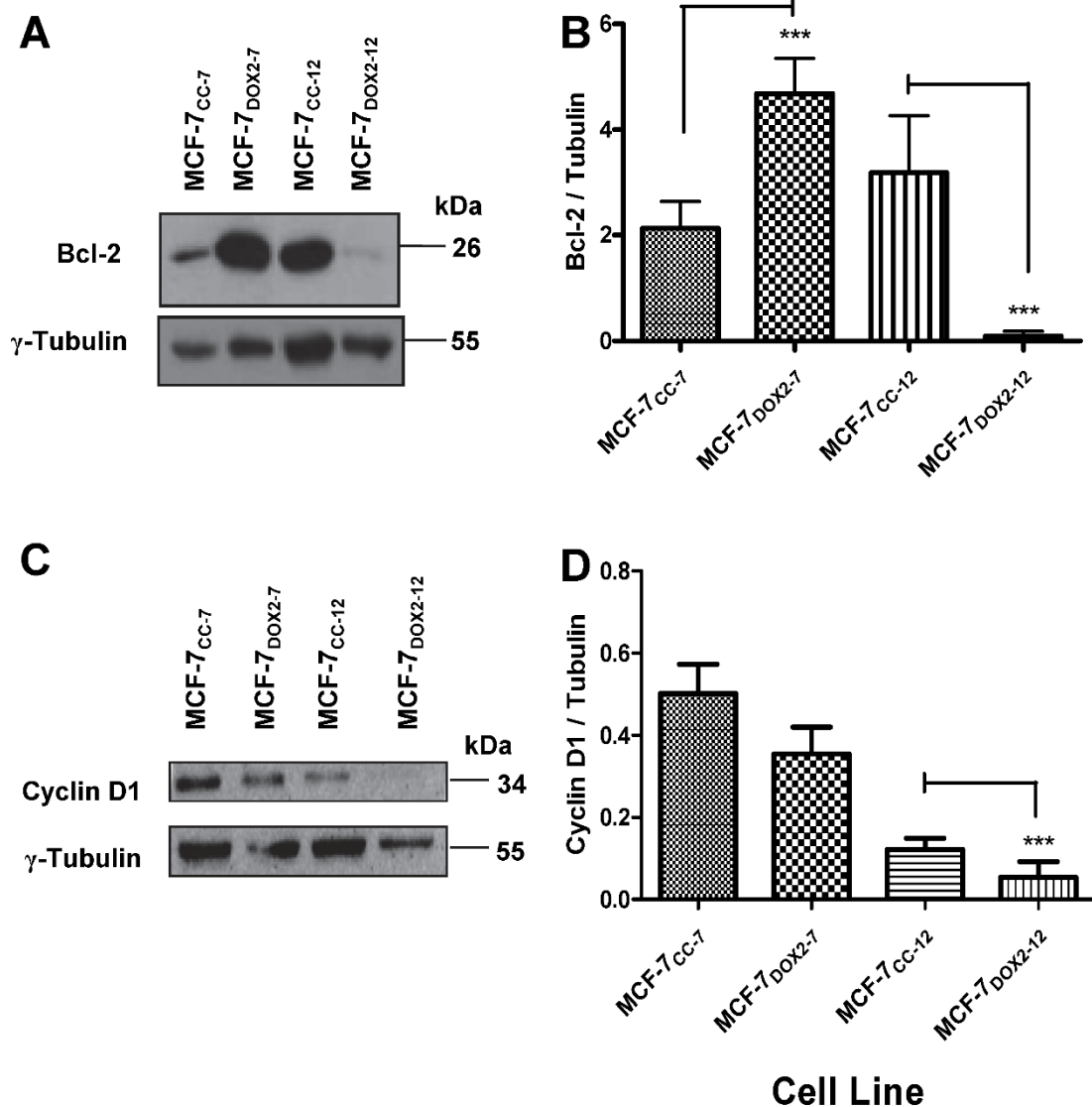


Figure 5-9 Expression of BCL-2, cyclin D1, and γ tubulin proteins in various cell lines, as determined in immunoblotting experiments with epitope-specific antibodies.

(A) A representative western blot for BCL-2 expression in MCF-7_{CC} and MCF-7_{DOX2} cells at selection doses 7 and 12. Results shown are representative of 3 independent trials. (B) Fold change in BCL-2 levels in MCF-7_{DOX2} cells (relative to the appropriate co-cultured control cell line). Results shown are the average of 3 independent trials. (C) A representative western blot for cyclin D1 expression in MCF-7_{CC} and MCF-7_{DOX2} cells at selection doses 7 and 12. Results shown are representative of 3 independent trials. (D) Fold change in cyclin D1 levels in MCF-7_{DOX2} cells (relative to the appropriate co-cultured control cell line). Results shown are normalized to the expression of γ tubulin and are the average of 3 independent trials. The significance of differences in expression levels for BCL-2 and cyclin D1 between samples was assessed using an ANOVA test, followed by Bonferoni testing. $n = 3$, *** $p < 0.001$

5.4.8: Knock-down of ER α Expression with Artemisinin

In order to assess the relationship between estrogen receptor signaling and the expression of estrogen-dependent genes involved in cellular growth and survival, we initially chose to knock down ER α expression using an siRNA approach. As shown in Supplemental**Figure 5-2**, the selected siRNAs only reduced ER α transcript expression by approximately half. This level of ER α transcript suppression had no significant effect on doxorubicin sensitivity (**Supplemental Figure 5-2**) or the expression of ER α -dependent genes. As an alternate approach, MCF-7_{CC} cells were treated for 72 hours with 300 μ M artemisinin. Artemisinin is a known potent blocker of ER α gene transcription and it would be expected that the expression of ER α -regulated genes such as Bcl2 and cyclin D1 would be strong affected by artemisinin (despite the lack of doxorubicin resistance in MCF-7_{CC} cells). Since artemisinin has been shown to be toxic to cells, we used the maximum concentration of artemisinin that had no effect on cell growth. **Figure 5-10 A** shows representative immunoblots for ER α , BCL-2, and Cyclin D1 in the absence or presence of artemisinin, with densitometry values for the expression of ER α , BCL-2, and cyclin D1 in various cell lines normalized to the expression of γ -tubulin depicted in **Figure 5-10 B, C and D**, respectively. As expected, artemisinin treatment resulted in a clear reduction in the expression of ER α and concomitant reductions in the expression of BCL-2 and cyclin D1 ($p < 0.001$ for all observations).

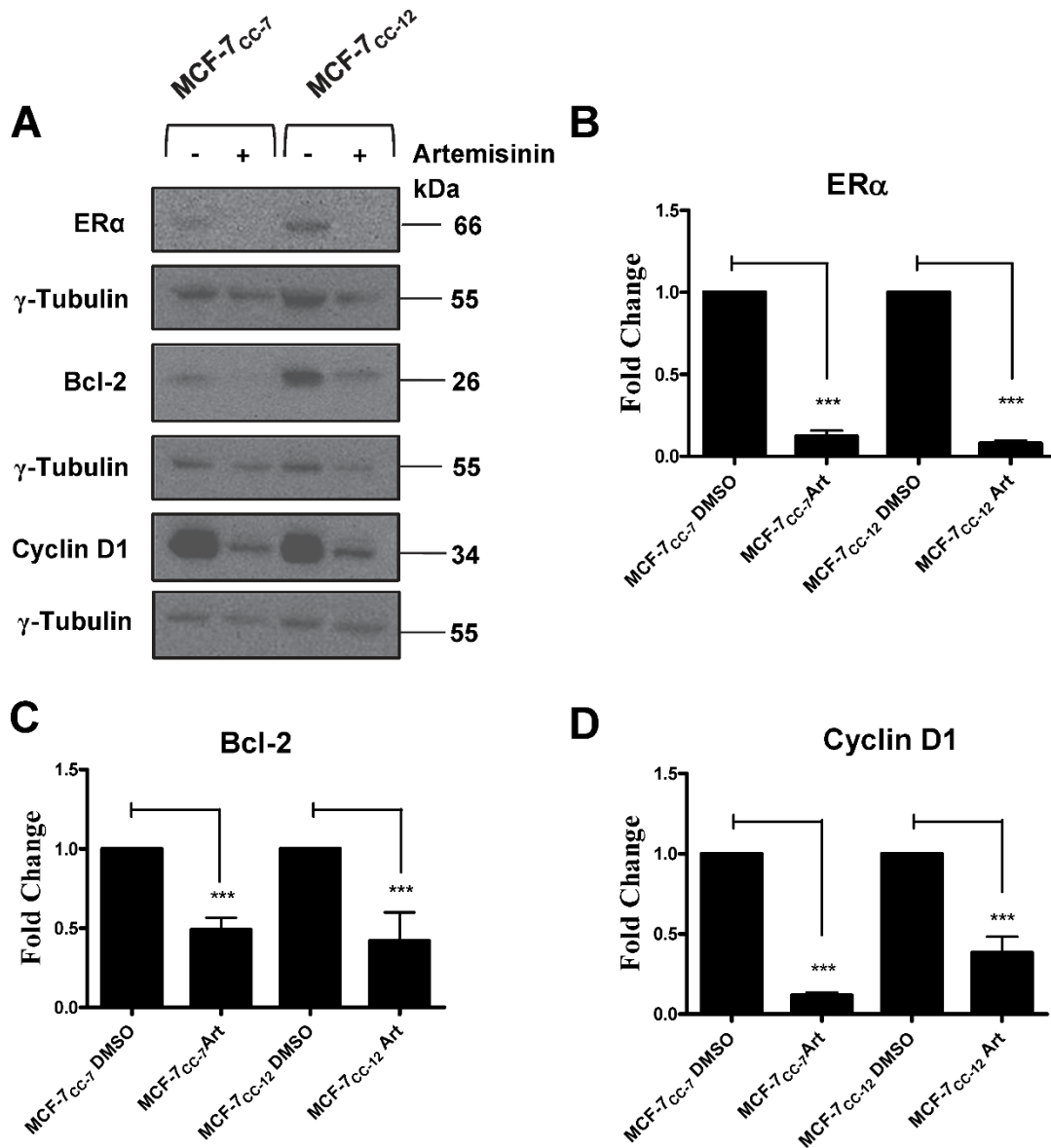


Figure 5-10 Artemisinin-mediated knockdown of ERα expression and its consequent effects on BCL-2 and cyclin D1 expression in MCF-7CC-7 and MCF-7CC-12 cells.

(A) MCF-7_{CC-7} and MCF-7_{CC-12} cells were treated with either DMSO or 300 μM artemisinin. Whole cell extracts of these cells were then monitored for BCL-2, cyclin D1, and γ tubulin protein expression using immunoblotting approaches with epitope-specific antibodies. γ tubulin was used as loading control. Blots are representative of 3 independent trials. (B) Fold changes in ERα levels induced by artemisinin in MCF-7_{CC} cells at selection doses 7 and 12. (C) Fold changes in BCL-2 levels induced by artemisinin in MCF-7_{CC} cells at selection doses 7 and 12. (D) Fold changes in cyclin D1 levels induced by artemisinin in MCF-7_{CC} cells at selection doses 7 and 12. All values depicted are the mean ± S.E.M. for 3 independent experiments. The significance of artemisinin-induced changes in the expression of the above proteins was assessed using an ANOVA test, followed by a Bonferoni correction. n = 3, *** p < 0.001

The changes in expression of cyclin D1 would be expected to have an influence on the proliferation rate of cells. To ascertain if doxorubicin-resistant cells had differing rates of cell division compared to wildtype cells, MCF-7_{CC-12}, MCF-7_{DOX2-12}, and MCF-7_{EPI-12} cells were plated in 10 cm plates at low densities (10^6 cells per plate) and allowed to grow until saturation. Cells were counted at daily intervals. It was observed that MCF-7_{CC-12} cells had a maximum growth rate of 180,000 cells h⁻¹, while MCF-7_{DOX2-12} and MCF-7_{EPI-12} cells had maximum growth rates of 130,000 cells h⁻¹ and 104,000 cells h⁻¹, respectively (**Figure 5-11**). Comparisons of curves generated by the Gompertz equation showed that the maximum specific growth rates differed significantly between all three cell lines ($p < 0.001$).

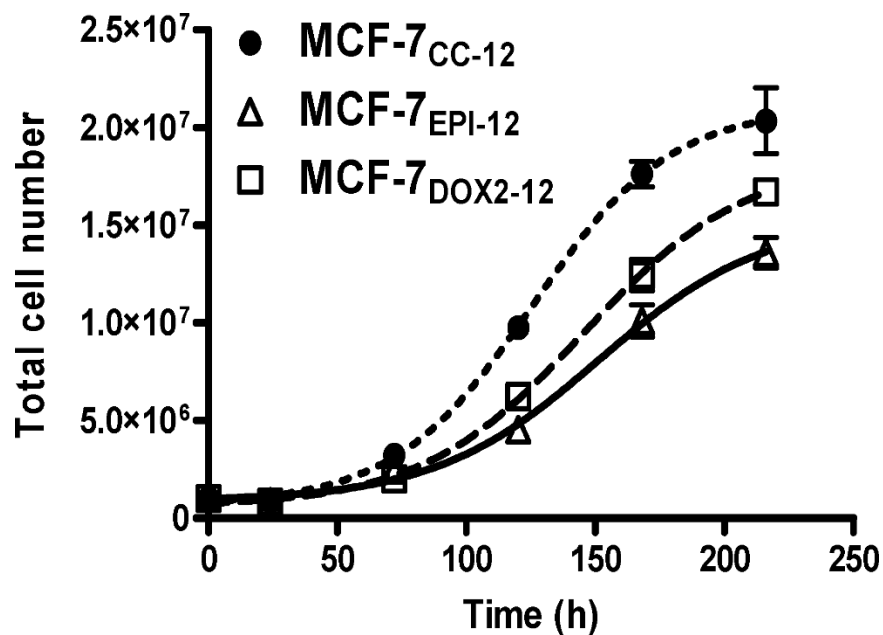


Figure 5-11 Cell growth curves for MCF-7_{CC-12}, MCF-7_{DOX2-12}, and MCF-7_{EPI-12} cells.

Exponentially growing cells were counted using a hemocytometer and introduced into T75 flasks at a density of 10^6 cells per 10 ml of D-MEM medium. Specific growth plots were generated using Graphpad Prism 5.0, modelled after the Gompertz growth equation. $n = 3$

5.5: Discussion/Conclusions

Resistance to cytotoxic chemotherapy drugs can be the result of several factors working in collaboration to protect tumor cells from drug-induced death. Previous microarray experiments comparing gene expression between the wildtype and the anthracycline-resistant MCF-7 cell lines used in this study (MCF-7_{DOX2} and MCF-7_{EPI} cells), revealed a number of gene expression changes associated with the acquisition of anthracycline resistance (13). Many of these changes in gene expression were subsequently validated using Q-PCR. Among the more prominent genes whose expression was altered upon acquisition of anthracycline resistance were members of the ATP-binding cassette (ABC) family of drug transporters as well as drug metabolizing proteins such as the AKRs. Coupled with findings from a previously published study (12) and a subsequent study (14), it became clear that two of the largest changes in gene expression for MCF-7_{EPI} cells were the *Abcb1* drug transporter (a known efflux transporter of epirubicin) and the aldo-keto reductase *AKR1c2* (or genes highly homologous to it). For MCF-7_{DOX2} cells, three of the largest changes in gene expression were the *Abcc1* drug transporter (a known efflux transporter of doxorubicin) and both *AKR1c3* and *AKR1b10*. The above ABC drug transporters would be expected to actively excrete anthracyclines from tumor cells, thereby reducing their accumulation in tumor cells and their cytotoxicity. Supporting this view, the uptake of doxorubicin into MCF-7_{DOX2-12} and MCF-7_{EPI-12} was found to be significantly lower than that of MCF-7_{CC-12} cells (12). The ability of the aldo-ketoreductases to hydroxylate and inactivate anthracyclines would also be expected to contribute to anthracycline resistance. However, *AKR1c3* has a clear preference for hydroxylation of idarubicin and daunorubicin over doxorubicin (49, 50). Moreover, while *AKR1C3* was able to hydroxylate doxorubicin as a

purified protein, these studies were unable to demonstrate the ability of AKR1c3 to hydroxylate doxorubicin within cells.

In the current study, we specifically examined the role that the overexpression of AKR proteins may play in E2 metabolism in MCF-7_{DOX2} and MCF-7_{EPI} cells and whether alterations in E2 metabolism may also contribute to the anthracycline-resistant phenotype in these cells.

5.5.1: AKR1c3 Expression and Function in Anthracycline Resistant MCF-7 Cells

In the present study, we confirmed the overexpression of AKR1c3 protein in MCF-7_{DOX2} cells, with MCF-7_{DOX2-11} cells having the highest expression of AKR1c3 gene and protein expression (**Figure 5-1 A**). This increase in AKR1c3 expression occurred upon acquisition of anthracycline resistance and the level of expression of AKR1c3 increased with increasing resistance to doxorubicin (**Figure 5-1 A**). Due to the relationship between increased AKR1c3 expression and elevated E2 synthesis from E1 (20, 51), we theorized that the higher cellular expression of AKR1c3 could potentially have broad impacts on E2-dependent signaling pathways that may also contribute to anthracycline resistance. Specifically, it was expected that the increased AKR1C3 expression would promote E2 synthesis from E1, which in turn would activate ER α -dependent survival pathways through either the non-genomic pathway via AKT or through the genomic pathway by increasing expression of BCL-2 (30, 52, 53). Such events would perhaps lead to enhanced survival and growth in the presence of anthracyclines (see further discussion below). While we observed that AKR1B10 levels in MCF-7 cells were dramatically increased upon selection for doxorubicin resistance (**Figure 5-1 A**), it is important to note that there is no published evidence that AKR1B10's promotes estrogen biosynthesis from estrone.

We subsequently demonstrated in this study that the increased expression of AKR1C3 in doxorubicin-selected cells (to dose level 12) did have a dramatic effect on the cells' ability to synthesize and secrete E2. As expected, cells that had higher levels of AKR1C3 expression also exhibited higher levels of basal and E1-induced E2 synthesis and excretion (**Figure 5-4 A**). This supports the idea that increased levels of AKR1C3 expression could be related to cell survival in chemotherapy resistant cell lines by promoting elevated production of E2 and subsequent activation of ER α -dependent growth and survival pathways, including the activation of AKT and the inhibition of apoptosis (30, 48, 54).

5.5.2: Overexpression of AKR1c3, by Itself, Does not Confer Anthracycline Resistance

To assess whether elevated expression of AKR1c3, by itself, could induce resistance to doxorubicin, we stably or transiently transfected MCF-7 cells with a vector for the constitutive expression of AKR1c3. Elevated levels of AKR1c3 expression relative to mock-transfected cells were observed in these transformants (**Figure 5-2**). Specifically, it was observed that transient transfection resulted in a greater induction of AKR1c3 protein expression (relative to mock transfected cells) than stably transfected cells (**Figure 5-2 C and D**). Clonogenic assays performed on stably selected clones showed on average of a 2.4 fold increase in resistance to doxorubicin relative to empty vector controls (**Figure 5-2 A**), but this difference was not found to be statistically significant in an ANOVA test. This increase in drug resistance was unlikely to be biologically relevant, since even a higher level of AKR1c3 induction in transiently transfected cells resulted in no change in doxorubicin sensitivity (**Figure 5-2 B**).

5.5.3: *AKR1C3* Knockdown in Doxorubicin-resistant Cells did not Restore Doxorubicin Sensitivity

Since overexpression of AKR1c3 transcripts and protein in MCF-7_{CC-12} cells showed little ability to induce doxorubicin resistance, we attempted to determine if elevated levels of *AKR1C3* and/or *AKR1B10* transcripts were required to maintain resistance in MCF-7_{DOX2-12} cells. Knockdown experiments were conducted using two different siRNAs targeting different regions of the *AKR1C3* or *AKR1B10* mRNAs. In addition, a scrambled control siRNA was used, to determine if any off-target effects and/or the process of transfection affected cellular sensitivity to doxorubicin. While individual transfections with the siRNAs resulted in as much as an 80% knockdown of AKR1c3 protein expression in the cells (**Figure 5-2**), no significant change in doxorubicin sensitivity was observed in the presence of either siRNA (**Figure 5-3 C and D**). To rule out compensatory effects with the individual knockdowns, we proceeded to knock down both AKR1c3 and AKR1B10 expression simultaneously by co-transfecting MCF-7_{DOX2-12} cells with both siRNAs. Knockdown with both siRNAs achieved the same level of knockdown of their respective RNAs as the individual transfections (up to 80%). However, no significant change in doxorubicin sensitivity was observed (**Figure 5-3 E**). To assess whether the effects of the *AKR1C3* and *AKR1B10* siRNAs are specific for their respective transcripts (with no effect on other AKR transcripts), we examined siRNA-transfected cells for expression of AKR1c3 and AKR1b10 proteins. We observed only minor changes in AKR1c3 levels in cells transfected with the *AKR1B10* siRNA (despite dramatically reduced AKR1b10 levels). Reduced expression of the target transcripts was verified in all siRNA experiments using western blotting experiments with AKR1c3 and AKR1b10 antibodies. Images in **Figure 5-2** are representative of these controls. The observation of a lack of effect of AKR1c3 knockdown on doxorubicin resistance suggests that the AKRs, while upregulated in doxorubicin resistance, did not actually

contribute to the drug resistant phenotype. Supporting this view, Hoffman et al. (49) observed that while AKR1c3 can convert doxorubicin to the less toxic doxorubicinol in a cell-free system, it has a relatively low affinity for doxorubicin and could not hydroxylate doxorubicin in *AKR1C3*-transfected cells. It should be noted that this is in contrast to studies involving the overexpression of an *AKR1C3* cDNA into cancer cells. A recent study by Zhong et al showed that overexpression of recombinant AKR1c3 in MCF-7 cells increased their resistance to doxorubicin (55). While our observations differ from these findings, we suggest that the differences observed are due to the level of overexpression. We were only able to increase the AKR1C3 level in MCF-7 cells by a maximum of 7-fold using Lipofectamine 2000. In contrast, Zhong et al. were able to overexpress AKR1C3 by nearly 200 fold over non-transfected cells using a viral expression vector (55). It is possible that there is a minimum threshold of overexpression required in order for AKR1C3 to promote estrogen production and/or have a significant impact on cell survival in the presence of doxorubicin. Moreover, this very high level of AKR1c3 overexpression may permit doxorubicin hydroxylation in cells, despite its poor affinity for doxorubicin as a substrate in cells (49, 50). The higher level of AKR1c3 expression in MCF-7_{DOX2} cells appears to be clearly sufficient to promote E2 biosynthesis and the activation of ER-dependent survival pathways. This may be particularly relevant at lower selection doses, where induction of the ABC transport proteins is minimal or much lower (12). We therefore postulate that in the MCF-7_{DOX2} cell model we present here, the AKRs contribute to the resistance phenotype. However, selection for survival in the presence of doxorubicin typically involves selection for multiple resistance mechanisms. Our previous studies have shown that MCF-7_{DOX2-12} cells strongly overexpress the Abcc1 drug transporter (12). Moreover, we have

recently observed that the Abcc1 inhibitor MK-571 can partially restore the sensitivity of MCF-7_{DOX2-12} cells to doxorubicin (Chewchuk et al., manuscript in preparation, Chapter 7).

5.5.4: E2 Biosynthesis and Cellular Growth Rates in Anthracycline-resistant Cells

We next determined whether the overexpression of AKR1c3 in anthracycline-resistant cells was associated with alterations in E2 biosynthesis and cellular growth rate. Stably transfected clones showed no significant difference in E1 to E2 conversion between cells transfected with an “empty vector” and *AKR1C3*-transfected cells (**Figure 5-4 B**). This suggested that the level of increase in *AKR1C3* expression in the stable transformants was insufficient to impact on E2 biosynthesis. Only a slight reduction in E1 to E2 conversion was observed when cells *stably* transfected with the *AKR1C3* expression vector were treated with β -cholanic acid, but not in the empty vector controls (**Figure 5-4 B**). This difference was statistically significant for clone MAT-32 using a Student t-test, but not by the more stringent ANOVA. However, β -cholanic acid was without effect when added to cells of the MAT-36 stable clone, which exhibited the highest expression level of AKR1c3 (**Figure 5-2 B and Figure 5-4 B**). Taken together our observations suggest that AKR1c3 overexpression is typically insufficient in the stable clones to promote additional E2 biosynthesis, which could be inhibited by β -cholanic acid.

When cells *transiently* transfected with the *AKR1C3* vector were assessed, no statistically significant increase in E2 biosynthesis was observed using an ANOVA or Student t-test (**Figure 5-4 C**), including cells transfected with an empty vector (mock-transfected cells). This was despite the increased expression of endogenous and FLAG-tagged recombinant AKR1c3 observed in these cells (**Figure 5-2 A**). However, a strong, highly significant reduction in E2

synthesis was observed upon treatment of empty vector- or *AKR1C3*-transfected cells with β -cholanolic acid (**Figure 5-4 C**). This was likely due to the effects of β -cholanolic acid on both endogenous and/or recombinant AKR1c3 expression observed during transfection of MCF-7 cells with the empty vector or *AKR1C3* expression plasmid (**Figure 5-2 A and C**).

In contrast to the above findings, E2 biosynthesis was considerably higher in drug-resistant MCF-7_{DOX2-12} and MCF-7_{EPI-12} cells than drug-sensitive MCF-7_{CC-12} cells. (**Figure 5-4 A**) This biosynthesis in the anthracycline-resistant cell lines was strongly inhibited by both the aromatase inhibitor letrozole and the aldo-keto reductase inhibitor β -cholanolic acid. The considerably higher levels of E2 production in the resistant cell lines are likely due to their higher level of overexpression of AKR1c3 compared to MCF-7_{CC-12} cells and MCF-7_{CC-12} cells stably or transiently transfected with the *AKR1C3* expression vector. These findings are consistent with those recently published by Byrns et al. (56). It may be possible to further increase the overexpression of AKR1c3 to more closely match the levels observed in the MCF-7_{DOX2} and MCF-7_{EPI} cells lines (possibly by other transfection methods such as electroporation or a retroviral vector. By increasing the overexpression of AKR1c3 to match more closely those of the chemotherapy drug resistant cell lines it may still be possible to induce doxorubicin resistance. However, more likely, the increase in AKR1c3 expression in transient or stable transfectants was insufficient to induce doxorubicin resistance.

5.5.5: Effects of Selection for Anthracycline Resistance on Cellular ER α Expression

E2 is a known promoter of growth in breast tumor cells (20, 25, 27). Thus, it would be expected that the elevated production of E2 in MCF-7_{DOX2-12} and MCF-7_{EPI-12} cells would result in higher growth rates for these cells relative to the co-cultured control cell lines. However, we

observed in this study a reduction in growth rates for MCF-7_{DOX2} and MCF-7_{EPI} cells compared to MCF-7_{CC} cells (**Figure 5-11**). It is therefore likely that some defect in the E2 signaling pathway exists in the anthracycline-resistant cells that prevents the elevated E2 levels from promoting growth. We thus looked at the levels of ER α and phosphorylated ER α (at Ser118) in the above cell lines in the absence or presence of estrogen and/or EGF. It has been reported that the phosphorylation of ER α on Ser118 is critical for the function of the non-genomic E2 signaling pathway, which regulates cell proliferation through effects on ERK and/or BCL-2 survival pathways (34, 43, 54). We observed that while EGF had no effect on ER α phosphorylation, MCF-7_{CC-12} cells treated with 100 nM E2 exhibited dramatic increases in ER α phosphorylation on Ser118 residues. Phosphorylation was substantially lower in MCF-7_{EPI-12} cells and undetectable in the MCF-7_{DOX2-12} cells (**Figure 5-6 A and B**). This was not expected since MCF-7 cells are normally ER positive and have been shown to require E2 signaling for normal growth (43). Subsequent immunoblotting experiments using an antibody to ER α revealed that the reduced phosphorylation was due to, at least in part, a downregulation of ER α expression in the anthracycline-resistant cell lines, in particular for MCF-7_{DOX2-12} cells (**Figure 5-6 A and C**). The reduced ER α expression appeared to be due to reduced levels of *ESR1* transcripts (**Figure 5-5**). The complete lack of detection of ER α phosphorylation for MCF-7_{DOX2-12} cells, suggests a complete downregulation of E2's ability to phosphorylate any available ER α as some ER α expression is still evident in these cells; see **Figure 5-6**.

We then hypothesized that upon selection for anthracycline resistance, the increased production of E2 via AKR1C3 overexpression activated a compensatory negative feedback loop to reduce ER α synthesis. However, subsequent experiments revealed that the reduction in ER α transcript levels occurred prior to AKR1C3 upregulation (compare **Figure 5-1 A** and **Figure 5-5**

A). This would argue against a negative feedback mechanism as the increase in E2 concentration would not have occurred to induce the progressive loss of ER α . The mechanisms for the reduced expression of ER α in MCF-7_{DOX2} and MCF-7_{EPI} cells relative to MCF-7_{CC} cells have yet to be elucidated, but may include: increased methylation of CpG islands or decreased histone acetylation within the *ESR1* promoter, resulting in reduced gene transcription (epigenetic changes), reduction in the activities of transcription factors associated with ER α expression such as the AP-1 transcription factors Fos and Jun (28), or reductions in ER α transcript stability.

It is interesting to note that changes in tumor ER α expression have been observed after neoadjuvant chemotherapy for patients with breast cancer, although the percentage of patients exhibiting a loss of tumor ER α expression post-chemotherapy was found to be only 6% (57). This is in contrast to 19% of patients losing tumor progesterone receptor (PR) expression after chemotherapy (57). While there are some clear limitations associated with this study of 368 non-randomized patients, it is possible that such losses in tumor ER α expression may be associated with resistance to anthracycline-based chemotherapy. The loss of tumor ER α expression status after neoadjuvant chemotherapy (if validated in future studies) would have very strong implications in terms of patient care. Patients with tumors lacking ER α expression post-chemotherapy would likely be unresponsive to endocrine therapies targeting estrogen signaling pathways, such as tamoxifen and exemestane (58, 59).

5.5.6: Effects of ER α Downregulation on the Growth of Anthracycline-resistant Cells

The growth rate of the anthracycline-resistant cells at selection dose 12 was assessed, since ER α has been documented to regulate cell growth in breast tumor cells (34). It was observed that both MCF-7_{DOX2-12} and MCF-7_{EPI-12} cells exhibited significantly reduced growth

rates in comparison to MCF-7_{CC} cells (Fig 11). This could be due to downregulation of ER α activity in these cells, although other pathways may also have been impacted on cellular growth rate. Despite this discrepancy, both cell lines exhibited lower growth rates which correlated with the loss of ER α expression and function. This observation led to the hypothesis that in acquiring resistance to chemotherapy, MCF-7 cells were selected for variants in the population with a slower growth rate. This could impart a survival advantage by allowing the cells more time to repair any cellular damage, before activation of cell death pathways. In addition, many chemotherapy agents such as doxorubicin selectively target rapidly dividing cells. In this light, it is reasonable to expect a growth promoting signaling protein, such as ER α , to be downregulated upon selection of breast tumour cells for anthracycline resistance.

5.5.7: Changes in the Expression of ER α -specific Genes upon Acquisition of Anthracycline Resistance

The reduction in ER α expression would be expected to strongly impact the expression of estrogen-dependent genes, in particular if this resulted in a change in the ability of the receptor to bind to the estrogen response element (ERE) regulating gene expression. To assess this, we first examined whether nuclear extracts from MCF-7_{DOX} cells exhibited a lower ability to bind the ERE than nuclear extracts from MCF-7_{CC} cells using the ER α TRANS Am kit. Nuclear extracts taken from MCF-7_{CC-7} and MCF-7_{DOX2-7} cells showed no statistically significant differences in the amount of active nuclear ER α (ERE binding) between the two cell lines. This was despite the reduced total levels of ER α in MCF-7_{DOX2-7} cells at this selection dose observed in western blotting experiments. This could indicate that while the total amount of ER α in the cells is reduced, the proportion of ER α that is active changes to maintain the basal requirements of the cells. However, MCF-7_{DOX2-12} cells showed a significant reduction in active ER α in nuclear

extracts relative to MCF-7_{CC-12} cells. This suggests that ER activity is also suppressed at high selection doses (consistent with the reduced phosphorylation of ER α), which involves more than the downregulation of ER α protein expression. Consistent with the abolished activity of ER α in MCF-7 cells, we observed reduced expression of two genes, whose expression is strongly upregulated by ER α , namely BCL-2 and cyclin D1 (30, 60) (**Figure 5-10**). Both genes possess EREs in their promoter regions (36), and have been shown to be highly responsive to E2 treatments (45, 48, 60). BCL-2 levels were significantly elevated in MCF-7_{DOX2-7} cells relative to co-cultured controls cell lines. As BCL-2 is an anti-apoptotic/pro-survival protein, it stands to reason that the elevated levels of BCL-2 would be observed early in the selection for doxorubicin resistance. However, it should be noted that in previous studies conducted in our laboratory (13), doxorubicin resistance was only achieved when the doxorubicin selection dose reached 29.1 nM (dose 9; MCF-7_{DOX2-9} cells). At selection dose 7 (6.5 nM doxorubicin), MCF-7_{DOX2-7} cells did not exhibit statistically significant resistance to doxorubicin, despite the elevated expression of BCL-2. It is possible that a low level of doxorubicin resistance (mediated by BCL-2) was acquired at selection dose 7, but this could not be detected due to the limitations in the sensitivity of the clonogenic assay. However, BCL-2 expression dramatically *decreased* during selection for resistance to higher doses of doxorubicin, co-incident with loss of ER α phosphorylation. While one would expect levels of an anti-apoptotic protein such as BCL-2 to remain high or increase further at higher selection doses, the levels of this protein actually decreased. There was simply insufficient active ER α to drive its expression. The downregulation of BCL-2 at higher selection doses would nevertheless activate another survival pathway (autophagy), which promotes the degradation of damaged organelles and greater survival from the generation of reactive oxygen species generated by doxorubicin (48). BCL-2 is a known inhibitor of autophagy through its

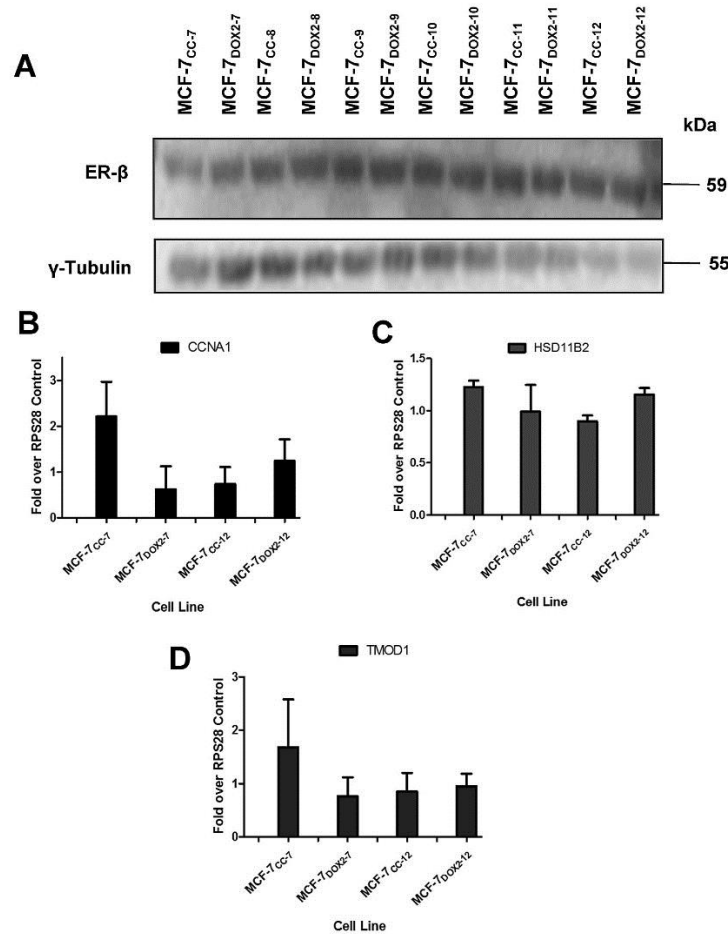
ability to bind Beclin 1 (reviewed in (61)). Since BCL-2 has been shown to be regulated by ER α (48), it stands to reason that ER α can act as an indirect regulator of the autophagic pathway. The downregulation of ER α activity would result in reduced BCL-2 expression, resulting in disinhibition (activation) of the autophagic pathway. In addition to changes in BCL-2 expression, cyclin D1 levels were observed to be reduced as active ER α levels fell. Since cyclin D1 promotes progression through the cell cycle, low cyclin D1 would be expected to slow cell cycle progression, permitting greater time for repair of doxorubicin-induced cellular damage.

The results from immunoblotting experiments suggest a clear correlation between the downregulation of ER α activity upon acquisition of doxorubicin resistance and changes in the expression of BCL-2 and cyclin D1. However, this correlation need not necessarily mean the relationship is causative. We thus examined the effects of Artemisinin on BCL-2 and cyclin D expression, since the drug is known to downregulate ER α expression, without inducing doxorubicin resistance (62). As expected when ER α levels were reduced due to treatment with Artemisinin, BCL-2 and cyclin D1 levels were also reduced as shown in previous studies. This suggests that the observed reduction in both cellular growth rate and saturation density as cells acquire anthracycline resistance may, in fact, be due to the loss of ER α protein levels and function, which in turn would reduce cell cycle progression and promote autophagic survival through negative effects on Cyclin D1 and BCL-2 expression, respectively.

We further show in this study that while AKR1c3 and AKR1b10 might be expected to contribute to doxorubicin resistance through the hydroxylation/inactivation of the drug or through its ability to combat reactive oxygen species generated by doxorubicin, downregulation of AKR1c3 and/or AKR1b10 does not result in the restoration of doxorubicin sensitivity in

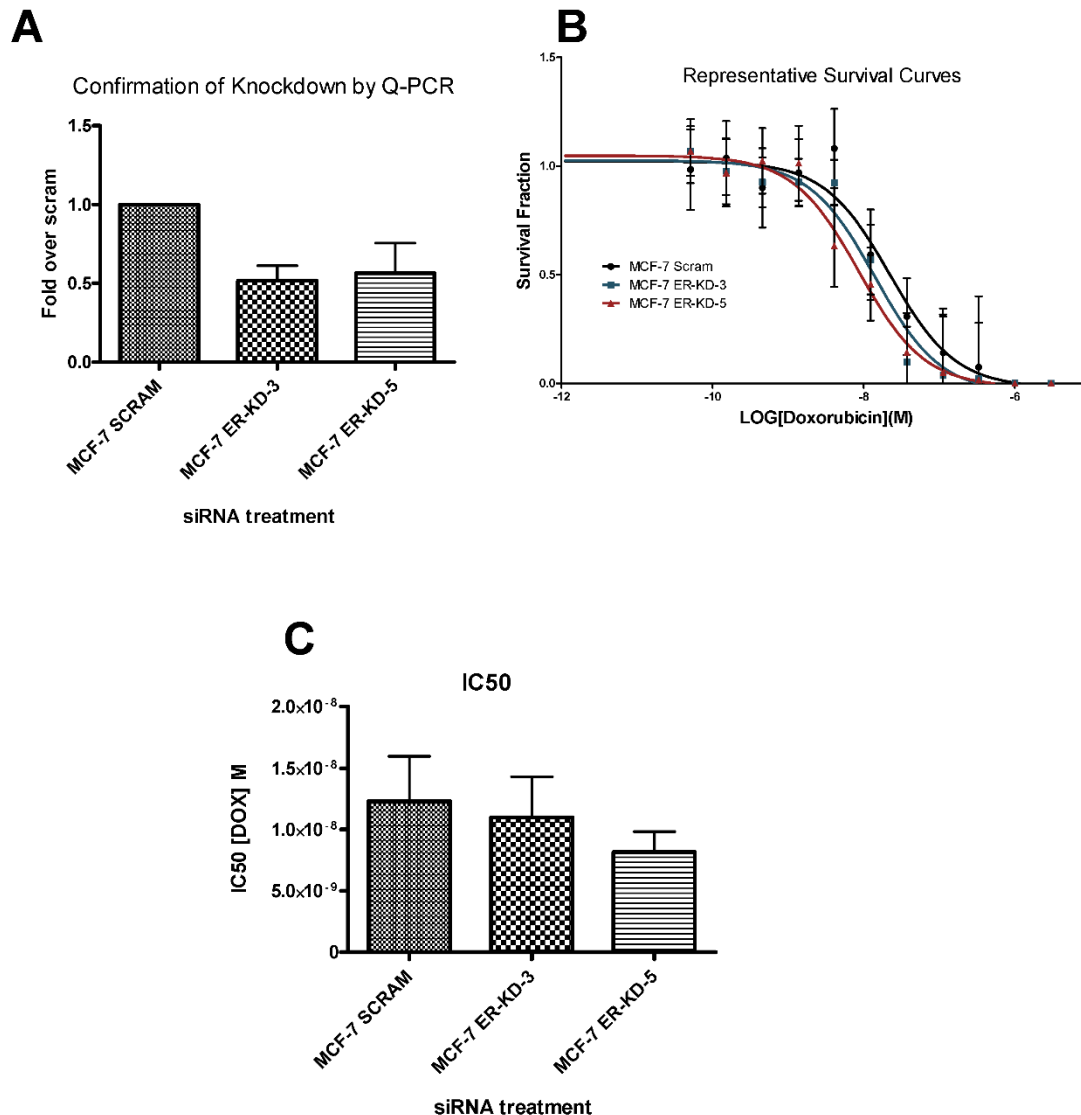
MCF-7_{DOX2-12} cells. In addition, others have shown that doxorubicin does not appear to be a good substrate for the above aldo-ketoreductases in cells (49, 50). However, we have also shown previously published studies that the aldo-keto reductase inhibitor β -cholanolic acid can almost completely restore doxorubicin sensitivity to MCF-7_{DOX2-12} cells (13, 14). This would suggest that β -cholanolic acid may have a wider effect on doxorubicin cytotoxicity than simply inhibiting the aldo-keto reductases, a hypothesis now supported by observations that β -cholanolic acid also promotes selective accumulation of doxorubicin into MCF-7_{DOX2-12} cells, but not MCF-7_{CC12} cells (Chewchuk et al., manuscript in preparation, Chapter 7).

5.6: Supporting information



Supplemental Figure 5-1 Analysis of ER-β activity in doxorubicin-resistant cells.

(A) Representative western blot of ER-β expression levels in dose selection 7–12 resistant and co-culture control MCF-7 cells. Primary antibody for ER-β was purchased from Santa Cruz Biotechnology (sc-8974). Blot is representative of 3 trials. (B–D) Q-PCR results for selected ER-β responsive genes in dose selections 7 and 12 with corresponding co-culture controls (n = 3), graphs represent fold expression relative to ribosomal protein S28 (RPS28) expression. Primers were purchased from Integrated DNA Technologies. (B) Relative expression of cyclin A1. Primer sequences for Cyclin A1 were F: 5'- GCA CCC TGC TCG TCA CTT G -3' R: 5'- CAG CCC CCA ATA AAA GAT CCA -3', (C) Relative expression of HSD11B2. Primer sequences for HSD11B2 were F: 5'- CTG GCT GCT TCA AGA CAG AGT -3' R: 5'- AGG CAG GTA GTA GTG GAT GAA -3' and (D) Relative expression of TMOD1. Primer sequences for TMOD1 were F: 5'- CCG GTT CCA GCG TCA CA -3' R: 5'- AGG AAA GGT CTG GGT TCC TAA GC -3'. No statistically significant changes were observed in overall protein levels of ER-β or in the expression of any of the tested ER-β responsive genes as a result of selection for resistance to Doxorubicin.



Supplemental Figure 5-2 Analysis of ER- α knock-down in MCF-7 wild type cells by siRNA.

siRNA was purchased from Life Technologies. Sequences correspond to catalogue numbers 4823 (ER-KD-3) and 4825 (ER-KD-5) (A) Q-PCR data for fold knock down of ER- α relative to scrambled control and was assayed in parallel with survival 24h post transfection. Graph is mean \pm SEM of 5 trials. (B) Representative clonogenic survival curve for MCF-7 cells with ER- α knockdown or scrambled control showing no significant shift in IC₅₀ associated with knockdown of ER- α relative to control. (C) Average IC₅₀ values of MCF-7 cells with siRNA knockdown of ER- α as derived from replicate survival curves. Graph represents the mean \pm SEM of 5 trials. No significant difference in IC₅₀ values was observed for either ER- α knockdown condition relative to scrambled control.

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Chapter 6

Additional Background/Rational for Investigation of Effects of β -Cholanic Acid on Chemotherapy Resistance

6.1: Introduction

In Chapter 5, we determined that while AKR's do function to increase the level of estrogen production in doxorubicin resistant cells (1), and they can contribute to metabolism of the drug (2), they do not directly contribute to the resistance phenotype in a statistically significant manner (1). It should be noted that this does not discount the role that drug metabolism can have on chemotherapy resistance. It is likely that in the model that was generated, other resistance mechanisms have a larger role in the resistance phenotype and thus overshadow the effects of metabolism. Regardless, an interesting observation was noted, resistance could be reversed by treating cells with β -cholanic acid (1–3). This observation was originally attributed to the known effects of β -cholanic acid as an AKR inhibitor. However since AKR knockdown and overexpression experiments failed to demonstrate changes in doxorubicin sensitivity, β -cholanic acid must also therefore have an alternate effect that would account for its ability to reverse resistance.

6.2: Bile Acids

β -cholanic acid, or ursodeoxycholic acid, belongs to a family of compounds known as bile acids. These compounds typically function to aid in digestion by emulsifying dietary fats to allow absorption through the small intestine (4, 5). In the body, bile acids are produced in the liver through metabolism of cholesterol (6). This process is carefully regulated through the action of various enzymes, including members of the AKR family (6). The overall process of bile acid synthesis will follow one of 2 routes known as the neutral and alternative pathways (7). The neutral pathway is centered around the metabolic action of the CYP7A1 protein which will begin with the hydroxylation of cholesterol (7). CYP7A1 is an enzyme with a very short half-life in hepatic cells and the expression of CYP7A1 is typically downregulated by most bile acids

allowing for very tight control of bile acid production through negative feedback (7). Once modified by CYP7A1, the modified cholesterol molecule proceeds through a series of 16 enzymatic reactions to form one of the two major bile acids found in humans: cholic acid and chenodeoxycholic acid. While the neutral pathway of bile acid synthesis is dominant in human hepatocytes, certain disease conditions lend themselves to activation of an alternative synthesis pathway (7).

Unlike the neutral pathway, which originates in the smooth endoplasmic reticulum, the alternative pathway begins in the mitochondria with the enzyme CYP27A1. Another key difference between the two pathways is the rate limiting step: while the neutral pathways is limited by hydroxylation of cholesterol, the alternative pathway is limited only by the availability of cholesterol transported to the mitochondrial membrane (7). This availability of cholesterol seems to be highly dependent on the expression of the cholesterol carrier StarD1 (7). Another major difference between these synthesis pathways is that unlike CYP7A1, which is only expressed in hepatocytes, CY27A1 is expressed in wide range of cell types throughout the body. With the broad range of cells that can make use of the alternative pathway, it is generally believed that the alternative pathway functions to regulate cholesterol levels in cells other than hepatocytes. In addition to synthesizing cholanic acid and chenodeoxycholic acids, the alternative pathway can also synthesize 25- or 27-hydroxycholesterol which can both function as regulatory oxysterols to control the expression of CYP7A1 by interacting with the Farnasoid X receptor in hepatocytes (7).

Once synthesized in hepatocytes, bile acids become further modified where they become conjugated with either a taurine or glycine molecule (8). They are then transported actively via a variety of different mechanisms to the gall bladder for storage where they are combined with

cholesterol and other phospholipids until they are needed to aid in digestion (4, 7, 9). Upon sensing digestible material entering the small intestine through the release of alimentary hormones, bile acids are secreted from the gall bladder into the lumen of the intestine at the duodenum where they combine with the digesting food. They will then activate various lipases in the digestive tract and solubilize lipids into micelles. These micelles, containing various fat soluble nutrients, are then taken up by enterocytes in the intestines. The bile acids themselves can then be reabsorbed by sodium-dependent transport proteins and transported back to the liver for recycling (7). Alternatively, the conjugated bile acids are digested by microbiota to remove the amino acid residues (8). During these processes most of the bile acids are transported back to the liver to undergo conjugation again completing the enterohepatic cycle (8). Very little of the bile acids will be found in circulating body fluids, and it is thought that what amounts are found there function primarily to regulate dissolved cholesterol levels throughout the body (10).

While the canonical role of bile acids as emulsifying detergents is well known and characterized, a growing body of literature has implicated bile acids as signalling molecules capable of binding the farnesoid X receptor (4). For instance, bile acids have been shown to play major roles in hepatocytes by increasing cell proliferation (of particular interest in cancer research), playing a role in liver regeneration, and liver fibrosis (7, 8). Many of these features seem to be related to the ability of bile acids to activate cell proliferative pathways. In the case of fibrosis, this can be both detrimental and beneficial, as lower bile acid concentrations can increase cellular regeneration of the liver, while very high levels of bile acids can be very detrimental and activate apoptotic pathways (4). In most cases, these high levels of bile acids only occur in disease states (liver disease, hepatitis infection, cirrhosis), and it should be noted that the best course of treatment for a build-up of secondary bile acids in the liver is ingestion of

ursodeoxycholic acid (5, 7, 8). Rather than the typical cholic and chenodeoxycholic acids found in normal human hepatocytes, ursodeoxycholic acid is produced in bears. When ingested by humans, ursodeoxycholic acid is known to promote secretion of bile acids from the liver, as well as counteracting the toxicities of secondary bile acids (7). Additionally ursodeoxycholic acid is known to activate alternative signalling pathways to cholic and chenodeoxycholic acids and prevent necrosis and apoptosis while also activating pro-survival pathways in human hepatocytes (4).

Given the broad range of effects of bile acids and the previous findings, as outlined in Chapter 5, we sought to determine how 5 β -cholanolic (ursodeoxycholanolic) acid could function to sensitize the MCF-7_{DOX2-12} cell line to doxorubicin. As was previously described, we established that 5 β -cholanolic could restore sensitivity in MCF-7_{DOX2-12} breast cancer cells, and that it did inhibit AKR1C3 function as predicted by previous publications. However upon closer examination of the AKR1C3 resistance mechanism, we determined that AKR1C3 was not playing a primary role in chemotherapy resistance, although it may contribute to resistance. The question remained as to how 5 β -cholanolic acid was reverting chemotherapy resistance. Previous assessments of the MCF-7_{DOX2-12} cells indicated that other resistance mechanisms could be playing a role. Among the most likely candidates is the ABCC1 drug transporter.

6.3: ABC Transporters

ABC proteins are a superfamily of proteins which are characterized by the presence of an ATP Binding Cassette domain. The members of the ABC family of proteins are transmembrane proteins which typically function to actively transport molecules across the cell membrane. These transport proteins are generally essential for moving molecules which do not easily

traverse the plasma membrane. This can include charged ions and other small molecules. The direction of transport (into or out of the cell) and the nature of the substrate transported are completely dependent on specific amino acid sequences known as substrate binding sites (11). While each member of the ABC protein family has very specific substrates to transport, they each do so in an energy dependent manner (12). The process by which ABC transporters perform their task is by active transport requiring ATP as an energy source. In addition to possessing an ATP binding cassette domain, all ABC transporter proteins are transmembrane proteins composed of a minimum of 1 transmembrane domain made up of a 6 pass transmembrane domain (11, 12). While most ABC transporters contain 2 such domains for proper function, members such as ABCC1 contain 3 transmembrane domains and ABCG2 contains only a single transmembrane domain. For the ABC transporters like ABCG2, which contain a single transmembrane domain, the proteins must form a dimer structure within the membrane to bring 2 domains into proximity with each other to function as an active transporter (11, 12).

As a brief overview of transporter function I will outline the general process by which a transporter can “pump” a substance out from the cell to the extracellular space. The process begins with the transporter in the “open” position in that the substrate binding site and the ATP binding cassette are exposed to the intracellular space (11). Once the appropriate substrate is bound to its domain, a conformational change occurs in the nucleotide (ATP) binding domain, increasing the affinity for ATP binding (11). The addition of ATP induces a further conformational change “closing” the intracellular domain and “opening” the core of the protein to the extracellular space. This conformational change then alters the affinity of the transporter to its substrate, releasing the substrate to the extracellular space (11). Once released, the ATP

molecules are hydrolysed to ADP which is then released and the transporter is restored to its original conformation (11). It should be noted that while this is a generalized mechanism for ABC transport, differences may exist and are debated depending on the specific ABC transporter in question (11).

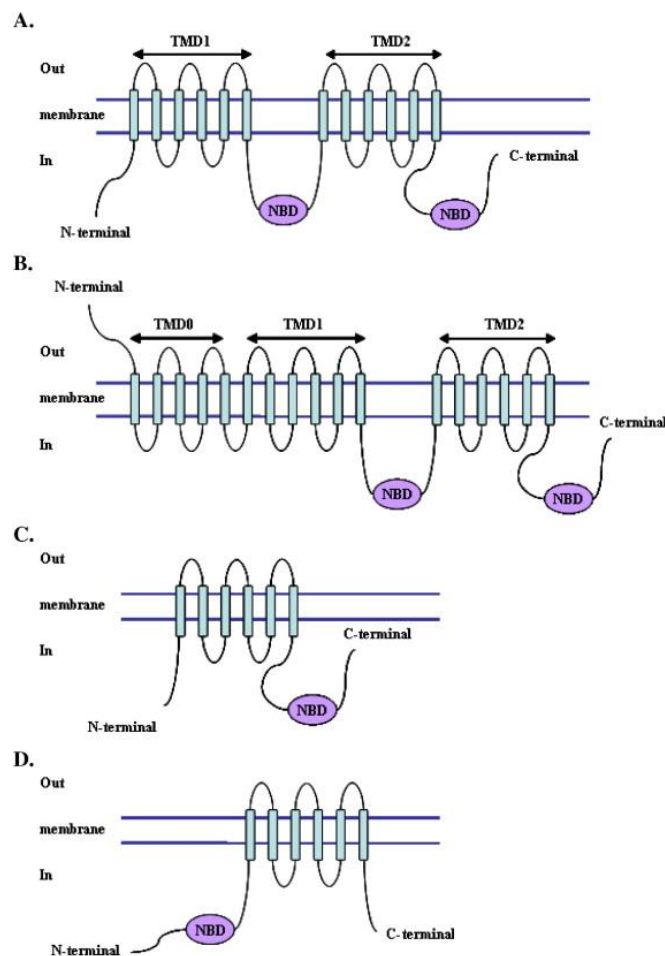
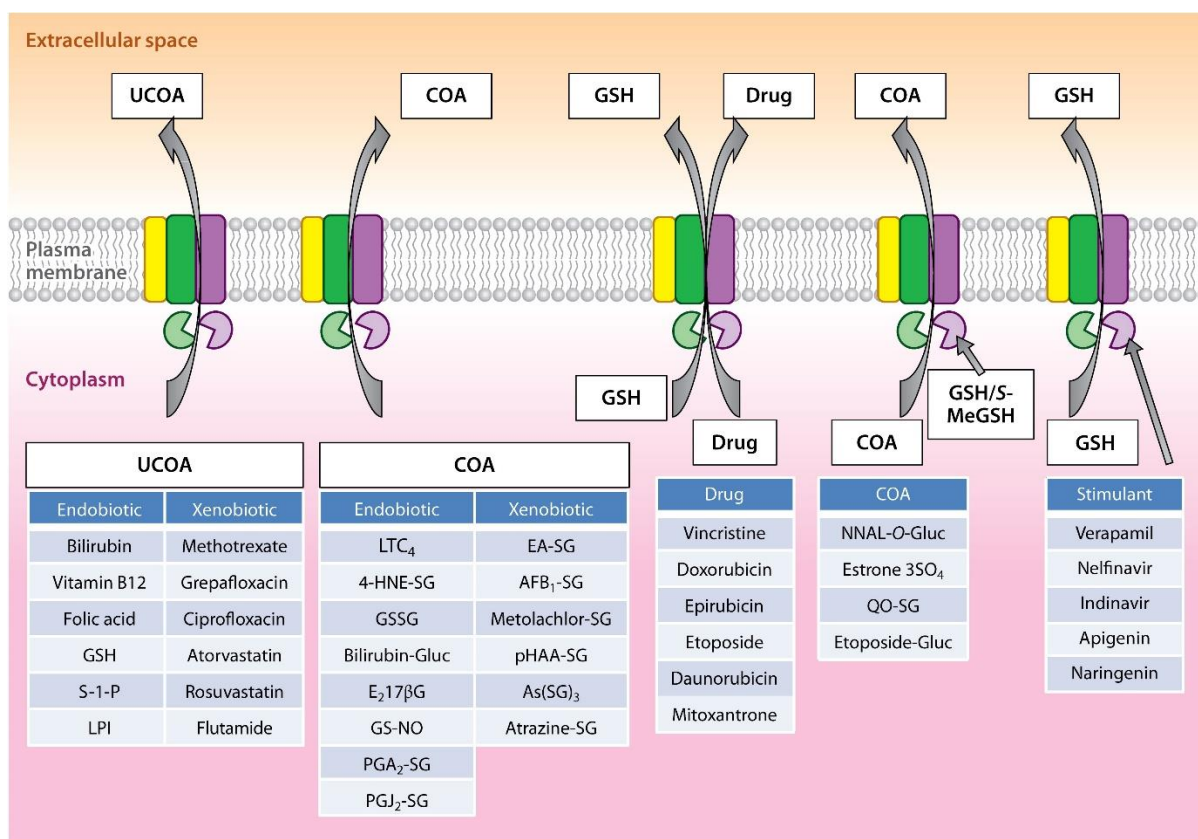


Figure 6-1 Structure of ABC transporters commonly associated with Chemotherapy resistance. Figure taken from Gillet et al. (13)

A. schematic structure of ABCB type transporters. B. Schematic structure of ABCC type transporters. C. Schematic structure of ABC half transporters. D. Schematic structure of ABCG type transporters.




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Figure 6-2 Schematic of common substrates and co-transported molecules of ABCC1 drug transporter. Taken from Col et al (14).

As has been stated, ABC transporters have a broad range of substrates that they can transport substrates in and out of the cell. Certain members of the ABC transporter family are of particular interest in the study of chemotherapy resistance. The ABC transporters most commonly associated with chemotherapy resistance are ABCB1, ABCC1, ABCC2 and ABCG2 (11, 15–18). Each of these ABC transporters has been shown to transport a variety of chemotherapy agents from the cell, removing the toxic agents before cellular damage can occur. Arguably one of the best understood transporters is ABCB1, also known as P-glycoprotein. This

transporter is normally expressed in a wide range of cells throughout the body, and has a very broad range of substrates that it can transport (19). In particular ABCB1 is found at high levels in hepatic cells, intestine, kidneys, and cells of the blood brain barrier where it serves to remove toxic substances and protect sensitive tissues (19). ABCG2 has a similar role in protecting cells from toxic compounds however its normal function is to remove toxic metabolites from dietary sources, specifically some of the breakdown products of chlorophyll (19). ABCC1 has a significant role in chemotherapy resistance, specifically with regard to the anthracycline class of chemotherapy agents. In general, ABCC1 functions as a defence mechanism for cells against xenobiotic agents which would normally be toxic to the cells. ABCC1 has a secondary role in normal cell physiology by regulating the levels of intracellular glutathione which is critical in mediating the cellular response to oxidative stress as well as the cycling of cysteine residues (14, 19). ABCC1 also plays an important role in regulating the inflammatory immune response in bone marrow-derived mast cells by secreting cysteinyl leukotriene (19). ABCC2 functions primarily to transport organic anions and secrete them into bile or urine for excretion from the body. ABCC2 can also transport various drug metabolites and conjugates to detoxify the body. ABCC2 is primarily expressed in liver and kidney cells where it can most easily facilitate the removal of these harmful substances. In addition to their normal functions, each of these ABC transporters has been seen to actively remove a variety of chemotherapy agents from cells. ABCB1 has the widest range of substrate specificity for chemotherapy agents among the drug transporters (20, 21). ABCC1 has a much narrower range of substrate specificity in relation to chemotherapy agents.

ABCC1 is unique as a chemotherapy transport protein in that in addition to ATP it also requires the presence of glutathione as either a co-transported molecule or a drug conjugate. It is

also of particular interest in the current study as it is the primary ABC transporter showing elevated expression in the MCF-7_{DOX2-12} cell line. ABCC1 is a potent transporter of doxorubicin and is therefore a likely candidate to be a primary mechanism of chemotherapy resistance in this cell line. We have also previously observed that treatment with bile acids is capable of reverting resistance in the MCF-7_{DOX2-12} cells.

6.4: Hypothesis and Specific Aims for Second Experimental Study

We hypothesize that in addition to their role in modulating AKR1C3 function, bile acids also have a regulatory role in ABCC1-mediated drug transport. To test this hypothesis, we propose to examine the following aims:

1. Monitor chemotherapy resistance in a variety of ABCC1 expressing cell lines including the MCF-7_{DOX2-12} cells, H69_{AR} (doxorubicin resistance small cell lung carcinoma) cells, and HEK293_{MRP1} transfected with ABCC1.
2. Monitor intracellular accumulation of doxorubicin in the aforementioned cells by flow cytometry.
3. Verify that ABCC1 is being inhibited by monitoring glutathione levels intracellularly.
4. Determine if the inhibition of chemotherapy transport is specific to ABCC1 or if ABCB1 can also be affected by bile acid treatment.
5. Determine the nature of β -cholanic acid-mediated ABCC1 inhibition.

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Chapter 7

Bile Acids Increase Doxorubicin Sensitivity in ABCC1-expressing Tumour Cells

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7.1: Authors' contributions

SC participated in writing of the manuscript and performed the majority of the described experiments. TB performed western blot analysis of ABCC1 expression. AMP devised and supervised the performance of the study, acquired grant funding to support the study, and helped write and revise the manuscript. He is also the corresponding author of this manuscript. All authors read and approved the final manuscript.

7.2: Abstract

Chemotherapy resistance is a complex phenomenon in cancer treatment, involving a broad spectrum of mechanisms to protect tumour cells from the cytotoxic effects of chemotherapy. Among the most studied mechanisms of drug resistance involve the overexpression of ABC transporters. These transporters facilitate the excretion of a variety of structurally distinct chemotherapy agents from the cytoplasm to the extracellular space. There has been considerable interest in the development of specific inhibitors for each of the ABC drug transporters. While inhibitors for specific ABC drug transporters have been developed, many of them affect the activity of more than one ABC transporter, particularly at elevated concentrations. It is also unclear whether they show clear efficacy for combatting resistance to specific drugs in tumour cells. In this study, we demonstrate the ability of two bile acids (β -cholanic acid (ursocholanic acid) and deoxycholic acid) to specifically inhibit ABCC1-mediated drug transport, augmenting doxorubicin accumulation into ABCC1-expressing but not ABCB1-expressing tumour cells. We also show that β -cholanic acid can restore sensitivity to doxorubicin in cells genetically engineered to overexpress the ABCC1 drug transporter and in cells that have acquired resistance to doxorubicin through the upregulation of ABCC1 expression.

7.3: Introduction

Cytotoxic chemotherapy agents are still widely used to treat human cancers in both the neoadjuvant and adjuvant settings (1, 2). While combinations of cytotoxic and targeted chemotherapy drugs can be effective in improving patient survival, a major impediment to this approach is the innate presence or acquisition of a wide variety of resistance mechanisms that circumvent the action of chemotherapy drugs (3).

Among the best described mechanisms of drug resistance are those associated with the elevated expression of one or more ATP-binding cassette (ABC) drug transporters (4). These transporters, namely ABCB1, ABCG2 and ABCC1 (5–7), play a role in normal cell function, as they regulate cellular levels of a variety of small endogenous molecules that include (but are not limited to) cholesterol, its derivatives, and a variety of chemical substrates (5, 6, 8, 9). The ABC transporters, especially ABCB1, also function at the blood brain barrier to protect the brain from exposure to toxic agents (10). Unfortunately, these transporters also circumvent the action of chemotherapy drugs by promoting the ATP-dependent efflux of drugs from the cytoplasm to the extracellular space (5). In contrast to their clear role in drug resistance *in vitro*, it remains unclear whether they play a critical or central role in clinical tumour resistance to chemotherapy agents in patients with specific cancers (11, 12). However, their involvement in drug resistance, disease progression and clinical outcome is becoming increasingly clear for haematological malignancies, such as Acute Lymphoblastic Leukemia (ALL) and Acute Myelocytic Leukemia (AML) (13, 14). The ABCB1 inhibitor, tariquidar, has been shown to increase sensitivity to doxorubicin, paclitaxel, etoposide, and vincristine in phase III trials of lung cancer. However, none of these trials were completed due to high systemic toxicity (15). This illustrates the limited utility of current ABC transporter inhibitors.

While many ABC transporters are thought to function in a similar fashion, their substrate specificities and tissue distribution vary greatly (5,6,16,17). ABCB1, also known as P-glycoprotein, is known to transport a large variety of commonly used chemotherapy agents, including the taxanes and anthracyclines (5). ABCC1, also known as MRP1, transports a variety of chemotherapy agents, but transports taxanes very poorly (16). One of the biggest challenges in combatting drug resistance mediated by ABC transporters is the lack of specificity of available inhibitors and/or their above-described toxic side effects on host tissues.

Bile acids are a class of chemical compounds derived from the metabolism of cholesterol (18,19). Their primary function is the solubilisation of fats and lipids during the digestive process (19,20). In general, bile acids are produced in the liver and transported to the gallbladder where they are stored until needed (18,19). The bile acids are then released into the small intestine where they form micelles with lipids. These micelles facilitate the absorption of lipids through the wall of the small intestine into the blood stream. Serum levels of bile acids vary greatly, from 0.3 μ M to up to 3 mM, depending upon the tissue or organ in which they reside (20,21). In addition to solubilizing lipids, bile acids also function as regulators of cholesterol metabolism by inhibiting enzymes of the aldo-keto reductase family. They also activate the farnesoid-X receptor, a transcription factor that regulates a variety of cellular functions (20, 22–25). While there is some evidence that bile acids can affect the expression of various ABC transporters through the farnesoid-X receptor (26, 27), in the present study we assessed the ability of bile acids to directly influence ABCC1 transport activity and ABCC1-mediated drug resistance. Previous studies have shown that membrane-perturbing agents and various bile acids such as deoxycholic acid and ursodeoxycholic acid can inhibit ABCC1-mediated transport of chemical agents in erythrocytes (28, 29). Since some bile acids are known to interfere with other

mechanisms of chemotherapy resistance (such as the ability of aldo-keto reductases to hydroxylate and detoxify doxorubicin), the prospect of targeting two or more resistance mechanisms simultaneously would be of interest in treating chemotherapy-resistant cancers (30, 31). Here we show the ability of β -cholanic acid, a known inhibitor of AKR1C3, to inhibit ABCC1-mediated drug transport. At concentrations normally used to inhibit AKR1C3, β -cholanic was able to inhibit ABCC1-mediated transport of doxorubicin. This same effect was not observed in cells where ABCB1-mediated drug transport is the primary mechanism of chemotherapy-resistance. We demonstrate that, even at high concentrations of deoxycholic acid and β -cholanic acid, doxorubicin retention is enhanced in chemotherapy-resistant cells that express ABCC1 but not ABCB1. This resulted in markedly improved doxorubicin sensitivity for the cells expressing ABCC1, but not for cells expressing ABCB1.

7.4: Materials and Methods

7.4.1: Cell Culture

The MCF-7 breast cancer cell line was originally purchased from the American Type Culture Collection and maintained in high glucose DMEM medium supplemented with 10% FBS and 100 μ g/ml streptomycin and 100 units/ml penicillin (Hyclone, Mississauga, Ontario) at 37° C in 5% CO₂. For subculturing, cells in T75 Sarstedt flasks were washed once with sterile PBS followed by the addition of 3 ml of a sterile 0.25% Trypsin, 10 mM EDTA solution (Invitrogen). MCF-7 cells were selected for resistance to increasing doses of either doxorubicin (MCF-7_{DOX2} cells) and characterized for expression of ABC transporters as well as other chemotherapy-resistance proteins by Hembruff *et al* and Veitch *et al* (30, 32). Doxorubicin-resistant cells were maintained in media supplemented with the corresponding selection dose of doxorubicin. Resistant cells were removed from drug-containing media 3-4 days prior to being used in

experiments. H69 small cell lung cancer cells, doxorubicin-resistant H69_{AR} small cell lung cancer cells, HEK293 human endothelial kidney cells, and *ABCC1*-transfected HEK293 cells (HEK293_{MRP1}) were kindly provided from Dr. Susan Cole of Queen's University, Kingston, ON, Canada. HEK293 and HEK293_{MRP1} cells were maintained under the same conditions as the MCF-7 cells with the exception that media was supplemented with 500 µg/ml of G418 to maintain selection for clones overexpressing *ABCC1*. H69 cells were maintained under similar conditions, except in RPMI growth media. Additionally, H69 and H69_{AR} cells grow as suspension cultures and consequently did not require the use of trypsin to subculture.

7.4.2: Doxorubicin Accumulation Assay

Steady-state doxorubicin retention in cells was measured via flow cytometry, since the drug is fluorescent. Cells were seeded onto 6 well plates at a density of 200,000 cells per well and allowed to adhere. Cells were treated with 2 µM doxorubicin, various concentrations of β-cholanic acid, deoxycholic acid, MK571, probenecid or a combination of these with doxorubicin for a period of 6 h. The concentration range for β-cholanic acid was varied in previous experiments to identify the maximally tolerated dose for MCF-7 cells (30, 32). Deoxycholic acid was tested at a concentration identical to β-cholanic acid, with no observable changes in cell health or morphology for either bile acid. Concentrations for MK571 and probenecid were based on the manufacturer's recommendation and also showed no discernible morphological effects on the cell population. Cells were collected using trypsin as described above and suspended in 1 ml PBS. Suspended cells were run on an FC500 flow cytometer (Beckman-Coulter) using an FL2 (575 nm) filter to detect doxorubicin fluorescence. Data points shown are the mean of 3 independent experiments and 10,000 events were measured in each experiment.

7.4.3: Glutathione Levels

Glutathione levels in MCF-7_{DOX2-12} whole cell extracts were measured using a glutathione quantification kit from ENZO Life Science, Inc. (Brockville, ON). Cells were plated in 10 cm plates at a density of 2×10^6 cells per plate. Cells were pre-treated with the glutamate-cysteine ligase inhibitor buthionine sulfoximine (50 μ M for 1h) to prevent subsequent glutathione synthesis. Cell lysates were collected and glutathione assayed as per the manufacturer's instructions. Data shown are mean of 3 independent experiments \pm SEM.

7.4.4: Clonogenic Assays

Cell viability was measured using a clonogenic assay as previously described (32). Briefly, cells were plated in 6 well plates at 200,000 cells per well. Cells were treated with increasing concentrations of doxorubicin in the presence or absence of test compounds for 24h. Cells were then collected and incubated in semi-solid methyl-cellulose medium until large colonies became visible, if possible (5-10 days). In each experiment, 12 random fields in each well were viewed and the number of colonies counted. IC50 values were calculated by determining the survival fraction relative to untreated control cells for each drug concentration. Each clonogenic experiment was repeated three times and thus the IC50 values reported are the means (\pm SEM) of three independent experiments.

7.4.5: Protein Isolation and Western Blot

Cells were grown on 10 cm cell culture plates (Sarstedt) and grown to 80% confluency. Cells were lysed and protein extracts prepared as previously described (33). Briefly, RIPA (Radio Immuno-precipitation Assay) buffer was modified to contain 1% SDS (w/v), 1x protease inhibitor cocktail (CompleteTM), 10 μ M sodium fluoride, and 2 mM sodium orthovanadate. This buffer was then used to lyse cells and solubilize proteins. Lysates were homogenized and sheared using a 21 gauge needle, and then subjected to centrifugation at 14,000 x g for 5 minutes at 4°C. The supernatant was then collected and aliquoted for western blotting. Protein content in the aliquots was assayed using a Pierce BCA protein assay prior to the addition of Laemmli sample buffer (1% w/v SDS, 0.25 M TRIS pH 6.8, 40% v/v glycerol, 0.133 M Dithiothreitol, 0.4% w/v bromophenol blue). The proteins in the samples were then denatured by boiling for 5 min, after which they were loaded on SDS PAGE gels.

ABCC1 protein levels were quantified by immunoblotting. First, 30 μ g of cell lysate was resolved on a 7% polyacrylamide gel. Proteins were resolved by electrophoresis at 90V, until the molecular weight range of interest (100-250kDa) was adequately distributed on the gel. The proteins were then transferred to nitrocellulose membranes using a semi-dry transfer apparatus applying 12V for 1 hour (Biorad). To detect ABCC1, membranes were first blocked using 5% skim milk in TBST (20 mM Tris pH 8.0, 137 mM NaCl, 0.1% (v/v) Tween 20) for 1 hour at room temperature. The membranes were then incubated overnight at 4°C in primary antibody (monoclonal QCRL-1 Santa Cruz Biotechnology, sc-18835) at a dilution of 1:200 in 5% skim milk in TBST. The secondary antibody (Santa Cruz Biotechnology goat anti-mouse HRP conjugate, sc-2005), at a 1:10,000 dilution in 5% skim milk in TBST, was applied the following day for 1 hour at room temperature. Images were captured on high-sensitivity film using

enhanced chemiluminescence reagent (Santa Cruz). Scanned images of the film were analyzed using AEaseFC 4.0 comparing pixel density levels of the bands relative to the background density of the film. Each band was normalized to a γ -tubulin loading control (Sigma, St. Louis, MO), with the same secondary antibody previously described.

7.4.6: Data Analysis

All graphs were prepared and statistical analysis performed using GraphPad Prism V5.0 software. All data points are representative of the mean \pm standard error of mean (SEM). Due to the multi-parametric nature of the data analysis, an ANOVA was chosen as the most suitable method of analysis. Analysis of Variance (ANOVA) tests were performed, assuming normal distribution for all datasets, followed by Bonferoni post hoc testing for significance. Comparisons were made between multiple points, i.e. between control cell lines and their respective chemotherapy-resistant cell lines at various selection doses, as well as comparing differences between treated and untreated cell lines or between transfected or non-transfected cell lines. Differences between samples or cell lines were determined to be significant when $P < 0.05$.

7.5 Results

7.5.1: Augmented Doxorubicin Accumulation by Bile Acids in Doxorubicin-resistant Cells

Since prior studies have shown that bile acids can inhibit ABCC1-mediated transport of chemical agents in erythrocytes (28, 29), we first assessed whether β -cholanic acid could enhance drug accumulation into breast tumour cells. Cellular doxorubicin levels were monitored

by flow cytometry, while taxane levels were quantified by liquid scintillation counting of (³H)-docetaxel in cells. These experiments were performed in a series of previously established cell line models known for their ABC transporter status (33,34). As shown in **Figure 7-1 A**, ABCC1-overexpressing doxorubicin-resistant MCF-7_{DOX2-12} cells exhibited no change in (³H)-docetaxel accumulation relative to cells “selected” in the absence of doxorubicin (MCF-7_{CC12} cells). For this reason, no further tests were performed to measure docetaxel uptake in these cells. In contrast, when MCF-7_{TXT10} cells (known to express ABCB1, but not ABCC1) were assessed for their ability to uptake doxorubicin, these cells exhibited just 50% of the doxorubicin accumulation of MCF-7_{CC12} cells (**Figure 7-1**). Interestingly, treatment of these cells with β -cholanic acid resulted in no statistically significant increase in doxorubicin accumulation (**Figure 7-1 B**). Similarly, the doxorubicin-resistant, ABCB1-expressing A2780_{ADR} ovarian cancer cell line exhibited only 4% of the doxorubicin uptake of parental A2780 cells and treatment of A2780_{ADR} cells with β -cholanic acid (at 200 μ M) also had no effect on their ability to accumulate doxorubicin. Tariquidar (100 nM) (an ABCB1-specific inhibitor) restored doxorubicin accumulation in A2780_{ADR} cells to 58% of parental A2780 cells. This illustrates the role of ABCB1 as a primary mechanism of resistance to doxorubicin in MCF-7_{TXT} and A2780_{ADR} cells and the utility of tariquidar (but not β -cholanic acid) as an effective inhibitor of ABCB1-mediated doxorubicin resistance (**Figure 7-1 C**).

In contrast to the above observations, β -cholanic acid (at a 200 μ M concentration) strongly restored sensitivity of ABCC1-expressing MCF-7_{DOX2-12} cells to doxorubicin and also increased doxorubicin accumulation (from 27% to 79% of drug-sensitive control MCF-7_{CC-12} cells). At 80 μ M, β -cholanic acid restored doxorubicin accumulation to just 41% of control cells). A comparable restoration in doxorubicin accumulation was also observed when MCF-

MCF-7_{DOX2-12} cells were treated with 200 μ M deoxycholic acid (to 76% of control cells). The small molecule ABCC1 inhibitor MK571 (80 μ M) was also effective at restoring doxorubicin accumulation to 65% of control cells, as expected. However, while MK571 is claimed to be a specific inhibitor of ABCC1, treatment of ABCB1-expressing MCF-7_{TXT10} cells with an equivalent dose of MK571 resulted in significant restoration of doxorubicin uptake (**Figure 7-1 B**). This cell line does not have detectable ABCC1 expression (33). The general organic anion transporter inhibitor probenecid (500 μ M) only partially restored doxorubicin accumulation in MCF-7_{DOX2-12} cells (to 45% of doxorubicin-sensitive MCF-7_{CC-12} cells). At 140 μ M, MK571 was more effective than 200 μ M β -cholanolic acid in restoring accumulation of doxorubicin (to 97% of control cells) (**Figure 7-1 D**).

Bile acids were also shown to be effective at restoring accumulation of doxorubicin into doxorubicin-resistant ABCC1-expressing H69_{AR} lung cancer cells, with deoxycholic acid being slightly more effective than β -cholanolic acid at restoring accumulation (95.5% and 87.8% of parental H69 cells, respectively) (**Figure 7-1 E**). As a control, probenecid also restored doxorubicin accumulation to 45% of parental cells.

To determine if the observed effects of bile acids on doxorubicin uptake were the result of inhibiting ABCC1 and not through effects on other molecules contributing to doxorubicin resistance in MCF-7_{DOX2-12} cells, HEK293 cells stably transfected with an expression vector for ABCC1 were also treated with doxorubicin and/or bile acids. In these HEK293_{MRP1} cells, doxorubicin accumulation was 34.4% of untransfected HEK293 cells (**Figure 7-1 F**). β -cholanolic acid (at 80 μ M and 140 μ M) augmented doxorubicin accumulation in HEK293_{MRP1} cells to 44.4% and 64.4% of untransfected cells, respectively (**Figure 7-1 F**). At 200 μ M, both β -cholanolic acid and deoxycholic acid increased doxorubicin levels in HEK293_{MRP1} cells to 75.2%

and 88% of untransfected cells, respectively. Probenecid (500 μ M), a general organic anion transport inhibitor, caused a restoration of doxorubicin accumulation to 61.5 % of untransfected cells.

ABCC1-mediated transport of doxorubicin has been shown to require the presence of glutathione (35). Consequently, we also measured glutathione levels in MCF-7_{DOX2-12} cells in the absence or presence of β -cholanic acid. Cells were first pre-treated with buthionine sulfoximine, which irreversibly inhibits glutathione biosynthesis, preventing cells from replenishing glutathione during the course of the experiment. As shown in

Figure 7-2, MCF-7_{CC12} cells showed no significant change in glutathione levels under any treatment conditions. In contrast, MCF-7_{DOX2-12} cells showed significantly increased levels of intracellular glutathione when treated with β -cholanic acid (1.9-fold higher than untreated MCF-7_{DOX2-12} cells). This same increase (1.9-fold) was observed in MCF-7_{DOX2-12} cells treated with both β -cholanic acid and doxorubicin.

7.5.2: Bile Acids Increase Sensitivity to Doxorubicin in Doxorubicin-resistant Cells

Since increased uptake of doxorubicin would be expected to increase the cytotoxicity of the drug, the effect of bile acids (β -cholanic acid or deoxycholic acid) on doxorubicin sensitivity in doxorubicin-sensitive and -resistant breast tumour cell lines (MCF-7_{CC12} and MCF-7_{DOX2-12} respectively) and lung cancer cell lines (H69 and H69_{AR}, respectively) was assessed using clonogenic assays. Each of the above drug-resistant cell lines express elevated levels of ABCC1. MCF-7_{DOX2-12} cells exhibited a significant increase in doxorubicin sensitivity in the presence of the bile acids with IC₅₀ values for doxorubicin of 790 nM for untreated cells and 170 nM and 98

nM for cells exposed to β -cholanic acid (200 μ M) and deoxycholic acid (200 μ M), respectively (Figure 7-3 F). Similarly, doxorubicin-resistant H69_{ADR} cells exhibited restored sensitivity to

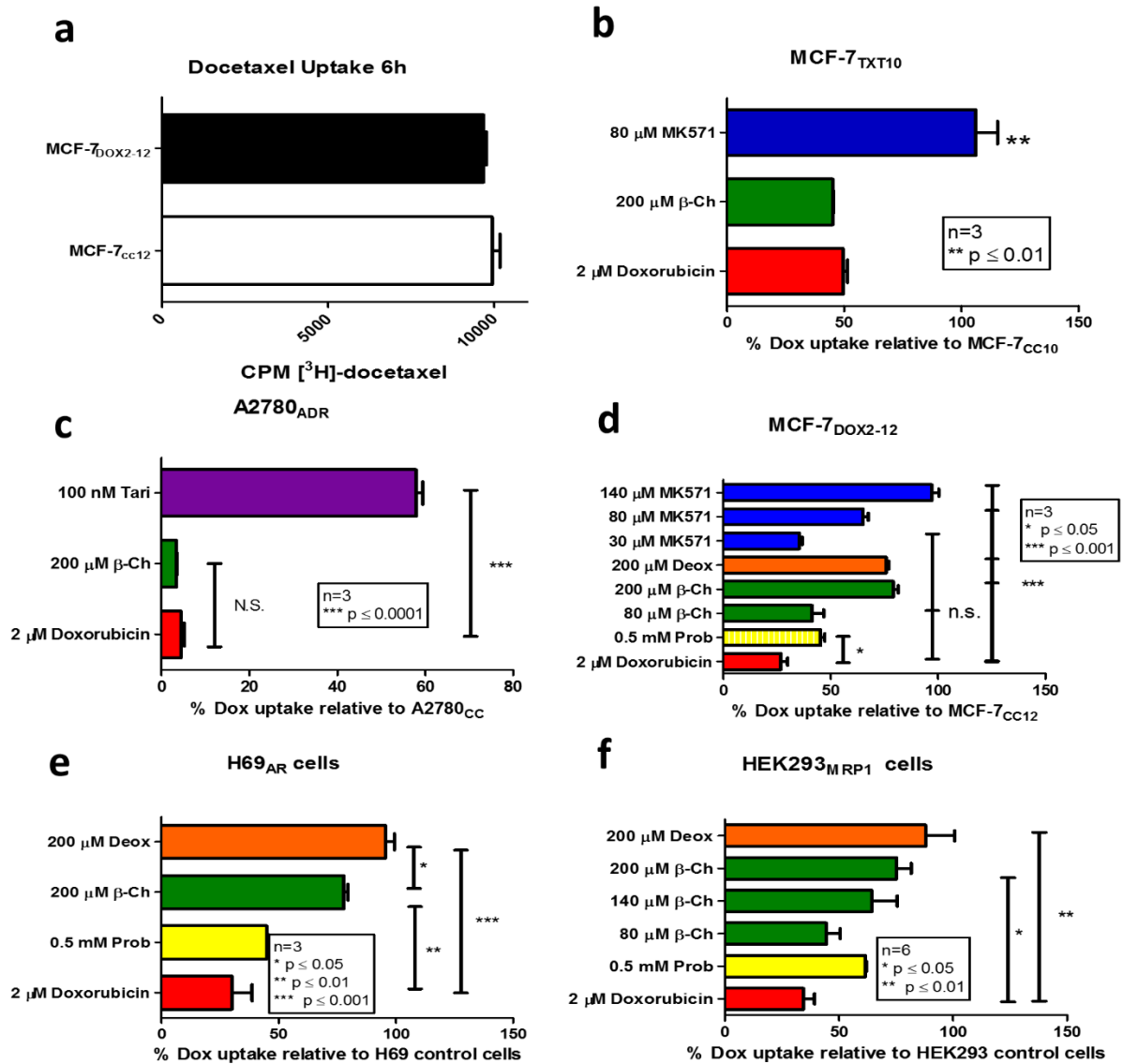


Figure 7-1 Measures of steady state uptake of doxorubicin or paclitaxel into various cell lines under various conditions. A. Tritiated docetaxel uptake into MCF-7_{CC12} and MCF-7_{DOX2-12} cells. B. Uptake of doxorubicin into MCF-7_{TXT-10} cells relative to MCF-7_{CC10} cells, as measured by flow cytometry. C. Uptake of doxorubicin into A2780_{ADR} cells relative to A2780 cells, as measured by flow cytometry. D. Effect of various agents on doxorubicin uptake into MCF-7_{DOX2-12} cells, relative to MCF-7_{CC10} cells. E. Effect of various agents on doxorubicin uptake into H69_{ADR} cells, relative to H69 cells. F. Effect of various agents on uptake of doxorubicin into HEK293 cells transfected with an ABCC1 expression vector relative to

untransfected HEK293 cells. Agents included β -cholanolic acid (b-ch, green), deoxycholic acid (deox, orange), MK571 (blue), probenecid (prob, yellow) or Tariquidar (tari, purple). All experiments were performed in triplicate, with p-values being calculated using ANOVA with Bonferoni post-hoc tests.

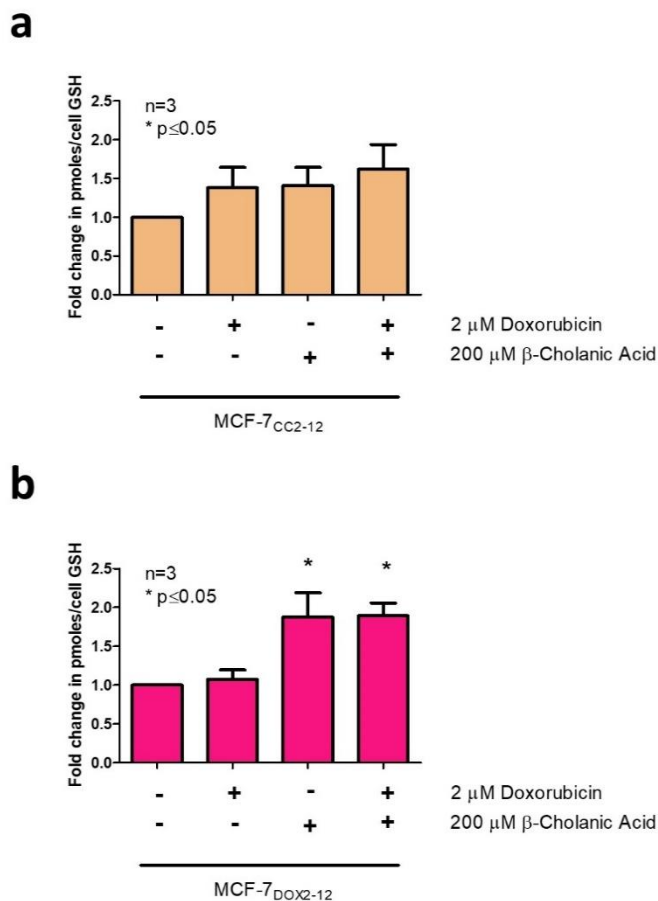


Figure 7-2 Effect of doxorubicin and β -cholanolic acid on glutathione levels in buthionine-treated MCF-7 cells.

MCF-7_{CC12} cells **A.** and resistant MCF-7_{DOX2-12} cells **B.** were treated simultaneously with buthionine sulfoximine (50 μ M for 1h) followed by combinations of doxorubicin (2 μ M) and β -cholanolic acid (200 μ M) for assessment of their glutathione levels. The data shows that treatment with β -cholanolic acid in MCF-7_{DOX2-12} cells results in elevated cellular glutathione levels. All data are standardized to respective untreated controls. Experiments were performed in triplicate. Statistical analyses were conducted using an ANOVA with a post-hoc Bonferoni correction.

doxorubicin with IC₅₀ values of 960 nM and 73 nM for untreated and β -cholanic acid treated cells, respectively (**Figure 7-3 E and H**).

To determine if the effects of β -cholanic acid on doxorubicin sensitivity were limited to cells selected for anthracycline resistance, ABCB1-expressing MCF-7_{TX_T10} cells were also treated with doxorubicin and/or β -cholanic acid. Unlike the above doxorubicin-resistant cell lines, MCF-7_{TX_T10} cells exhibited no change in doxorubicin sensitivity (data not shown). This suggested that β -cholanic acid does not potentiate doxorubicin cytotoxicity in all drug-resistant cell lines and may demonstrate selectivity towards cells overexpressing ABCC1. Hembruff et al. showed that MCF-7_{TX_T} and MCF-7_{EPI} resistant cells express high levels of ABCB1, while MCF-7_{DOX2-12} cells express high levels of ABCC1 transporter (33). These cell lines were developed in our laboratory by selection for survival in increasing drug concentrations and all are isogenic with parental MCF-7 cells and cells “selected” in the absence of drug to control for increasing passage number (MCF-7_{CC} cells). MCF-7_{DOX2-12} cells, however, do not express the very high levels of ABCC1 seen in H69_{AR} cells (**Figure 7-4**). Thus, the differing levels and types of drug transporters in the above drug-resistant cell lines may account for the differing ability of β -cholanic acid to increase cellular sensitivity to doxorubicin. Moreover, β -cholanic acid may selectively affect cells overexpressing ABCC1, given that ABCB1-overexpressing doxorubicin-resistant A2780_{ADR} cells did not exhibit increased doxorubicin sensitivity in the presence of β -cholanic acid, while ABCC1-overexpressing doxorubicin-resistant ABCC1-overexpressing cells did. To test this hypothesis, HEK293 cells and HEK293_{MRP1} cells were examined for their sensitivity to doxorubicin in the absence or presence of bile acids. These cell lines only differ in their expression of ABCC1. As seen in **Figure 7-3 C and E**, both β -cholanic acid and

deoxycholic acid augmented doxorubicin cytotoxicity in HEK293_{MRP1} cells. HEK293_{MRP1} cells exhibited an IC₅₀ for doxorubicin of 47 nM in the absence of bile acids and IC₅₀ values of 12 nM and 6.6 nM in the presence of β -cholanic acid and deoxycholic acid, respectively.

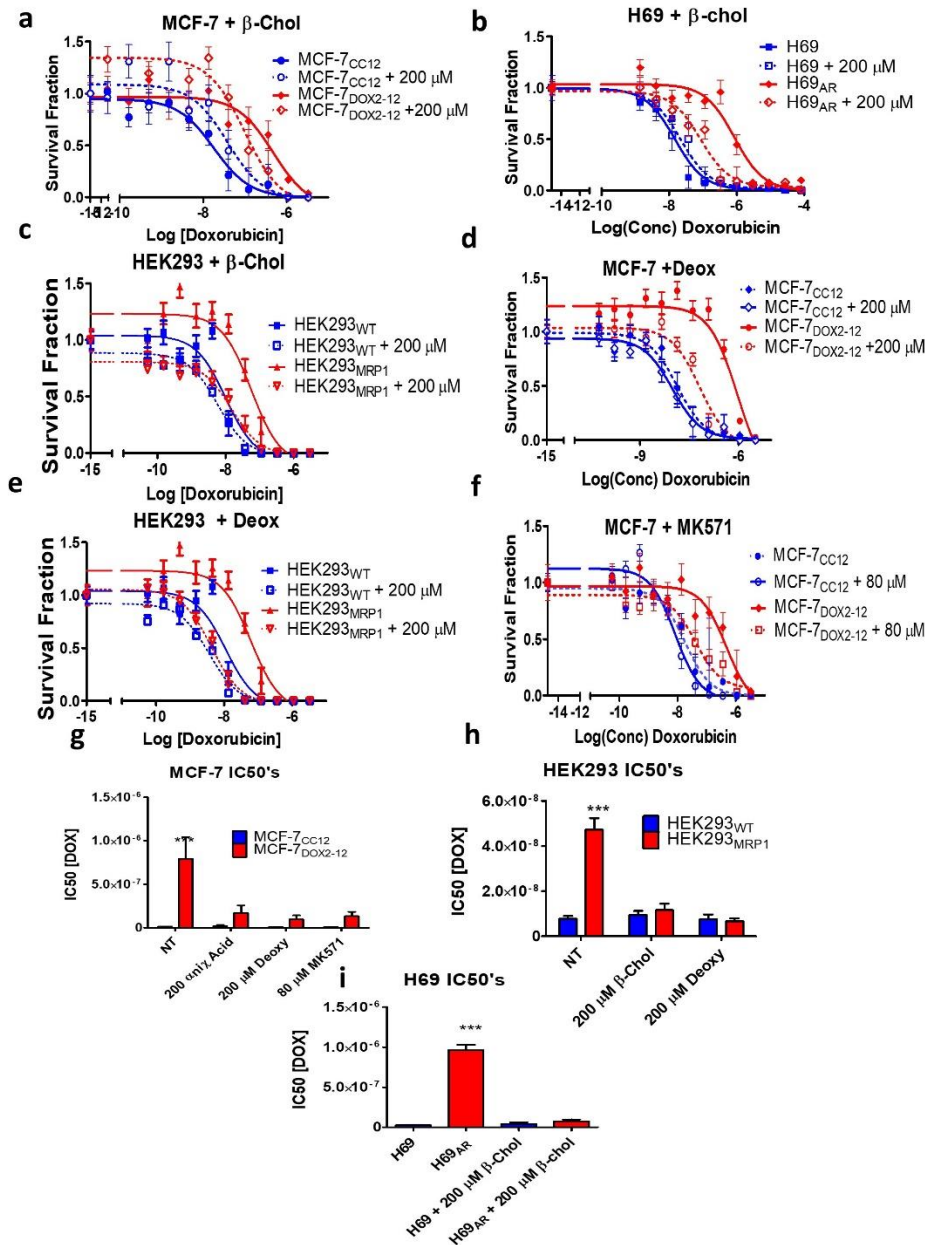


Figure 7-3 Effect of bile salts on sensitivity of human tumour cell lines to doxorubicin as measured by clonogenic assay.

The cell lines used were MCF-7_{CC12}, MCF-7_{DOX2-12}, H69, H69_{AR}, HEK293 and HEK293_{MRP1} cells. **A-C.** show survival curves for MCF-7, H69 and HEK293 (with respective doxorubicin resistant counter-parts) cells treated with increasing concentrations of doxorubicin in the presence or absence of 200 μ M β -cholanic acid. **D-E.** Doxorubicin sensitivity curves for MCF-7 and HEK293 cells as a function of doxorubicin concentration in the presence or absence of 200 μ M deoxycholic acid. **F.** Effect of MK571 on doxorubicin sensitivity in wildtype and doxorubicin-resistant MCF-7 cells. **G-I.** Summary of IC₅₀ values for doxorubicin in for various

cell lines under various conditions. *** $p < .001$ as determined by ANOVA analysis with a Bonferoni correction.

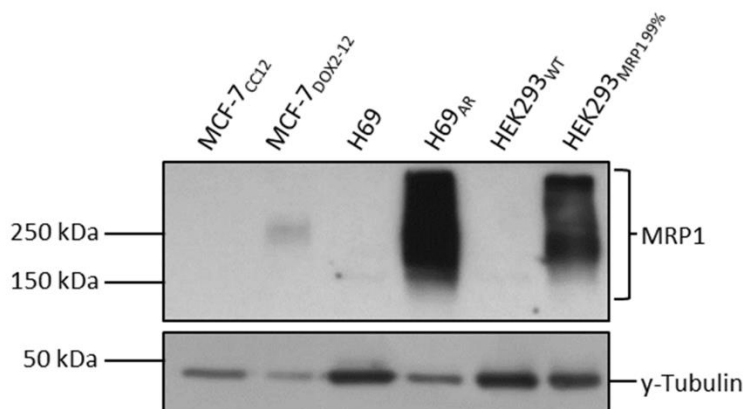


Figure 7-4 Representative western blot of ABCC1 levels in MCF-7 sensitive and doxorubicin resistant cell lines

H69 and H69AR cell lines, and HEK293 parental and ABCC1 transfected cell lines.

7.6: Discussion

In this study, we provide strong evidence that the bile acid β -cholanic acid is an effective and specific inhibitor of ABCC1-mediated doxorubicin efflux. This results in a strong promotion of doxorubicin sensitivity in doxorubicin-resistant tumour cells, providing they express the ABCC1 drug transporter. Previous studies by our laboratory and others have shown that β -cholanic acid is also an effective inhibitor of aldo-keto-reductase 1C3, which can promote resistance to anthracyclines by inducing their hydroxylation (30, 32, 36). However, potentiation of doxorubicin cytotoxicity by β -cholanic acid in our study did not appear to be through inhibition of drug hydroxylation because another bile acid (deoxycholic acid), which has no aldo-keto reductase inhibitory activity (37), and was able to potentiate doxorubicin cytotoxicity in our study at concentrations identical to β -cholanic acid. It should be noted that the potency of bile acids is approximately 2.5-fold lower

than a well-known inhibitor of ABCC1 activity, namely MK571. These are, nevertheless, well within the physiological range of bile acids in humans (19). Moreover, while MK571 was more potent than bile acids at inhibiting ABCC1-mediated doxorubicin efflux from tumour cells in our study, our findings also show that at a lower but equally effective concentration of 80 μ M, MK571 also inhibited doxorubicin efflux from docetaxel-resistant MCF-7_{TXT10} cells, which express ABCB1 and ABCC2 as their predominant drug transporters (**Figure 7-1**) (33). Consistent with our findings, Molinas *et al.* showed that MK571 as well as cyclosporine A and probenecid were able to inhibit ABCB1 transport activity (17). Probenecid, another inhibitor of ABCC1 was also able to partially inhibit ABCC1 transport but at a concentration more than double that of β -cholanolic acid, suggesting that β -cholanolic acid is a more potent inhibitor than probenecid in the presented model.

While bile acids appear to be specific for inhibition of ABCC1 over ABCB1, it should be noted that we did not examine the possible effects of bile acids on other ABC transporter proteins. Since the MCF-7_{TXT10} cell line used was previously shown to express both ABCB1 and ABCC2 (33), we cannot rule out the ability of β -cholanolic acid to inhibit ABCC2 transporter activity. The restoration of doxorubicin uptake and cytotoxicity in cells overexpressing exogenous ABCC1 (HEK293_{MRP-1} cells) further suggests that the effect of bile acids in sensitizing cells to chemotherapy is primarily due to their ability to affect ABCC1 transport and not through potential additional effects on other proteins implicated in doxorubicin resistance.

The selectivity of bile acids for inhibition of ABCC1 transport activity (with little effect on ABCB1-mediated drug efflux) is noteworthy, since current ABCC1 inhibitors do not exhibit strong selectivity for drug transporters. For example, cyclosporine A inhibits a wide variety of ABC transporters (8, 9, 13) and, as demonstrated in this and another study (17), MK571 also

inhibits ABCB1-mediated drug efflux. In contrast, our current study suggests that β -cholanolic acid may have greater specificity, with no effect on doxorubicin accumulation in ABCB1-expressing cells. It thus warrants further examination. This would include assessing the efficacy of bile acids to augment efflux of a variety of chemotherapy drugs in chemoresistant cells, including cell lines genetically engineered to overexpress a specific ABC transporters. In particular, expression vectors should include ABC transporters previously implicated in chemotherapy resistance and drug disposition, including additional ABCC isoforms (38) and ABCG2 (39).

Our study also showed that bile acids have no effect on doxorubicin accumulation into drug-sensitive tumour cells that do not express ABC drug transporters. This is important, since bile acids are known to affect lipid solubility (19) and this could increase plasma membrane permeability towards doxorubicin and other chemotherapy agents tumour.

While in some cases, cells treated with very low doses of doxorubicin did exhibit high survival fractions than their untreated counterparts, this is likely an artifact of the clonogenic process. While care is taken to ensure equal plating of cells between samples, in some cases cells may get spread to the periphery of the plate where colonies cannot be distinguished clearly, or in some cases, plating efficiency can vary within an experiment. For this reason, only representative clonogenic experiments were presented, while IC₅₀ values were calculated as an average of 3 or more independent clonogenic studies.

Hembruff *et al* (33) demonstrated that a majority of MCF-7 cells selected for resistance to anthracyclines and taxanes expressed ABCB1, including cells selected for resistance to docetaxel, paclitaxel or epirubicin. Docetaxel-selected cell lines also expressed ABCC2 at low selection doses (Cells exposed up to an intermediate selection dose were used in our study

(MCF-7_{TX10} cells)). In contrast, the doxorubicin-resistant cells used in our study predominantly expressed the ABCC1 transporter, with no elevated expression of ABCB1 (33). Since only our MCF-7_{DOX2-12} cell line showed reductions in doxorubicin accumulation in the presence of bile acids, we therefore chose to focus on ABCC1 rather than ABCB1, which is not expressed in MCF-7_{DOX2-12} cells. In addition, the docetaxel-resistant cell line used in our study overexpresses both ABCB1 and ABCC2 (MCF-7_{TX10} cells) and did not show any changes in doxorubicin-uptake when treated with β -cholanic acid. This suggests that β -cholanic acid also has no effect on ABCC2-mediated drug transport and/or that ABCC2 does not contribute sufficiently to the doxorubicin-resistant phenotype.

We proceeded to further test our hypothesis using two other ABCC1 expressing cell lines, the doxorubicin-resistant H69_{AR} lung cancer cell line, and an ABCC1-transfected cell line (HEK293_{MRP1}). For both cell lines, high concentrations of bile acids reduced the IC₅₀ values for doxorubicin by 3-4 fold compared to their respective parental controls. Similar to what was observed in parental MCF-7 cells, bile acids had no effect on doxorubicin sensitivity in parental H69 and HEK293 cells. We do note that complete restoration of sensitivity is not achieved in the MCF-7 cells as compared to the other cell lines, despite the lower expression level of ABCC1 in the MCF-7 cells as compared to the H69 or HEK cells. We suggest that this may be due to other, yet undefined, resistance factors which would contribute to the MCF-7 resistance phenotype.

One of the major obstacles in studying the regulation and function of ABC transporters and their contribution to drug resistance in tumour cells is that they cannot be studied in complete isolation of the cell. The transport activity of these molecules, however, can be studied by creating micelles derived from cells engineered to express specific transporters. These micelle models have the benefit of isolating a specific transporter and measuring its ability to actively

transport fluorescent substrates into micelles. While such approaches can accurately measure transport kinetics, a full tumour cell line model is preferred when studying the contribution of drug transporters to drug resistance in cells and the ability of ABC transporter inhibitors (such as bile acids) to augment drug sensitivity. This is because a variety of molecules, proteins, and cellular organelles impact on tumour cell sensitivity to chemotherapy.

Previous studies have shown that bile acids can inhibit ABCC1 transport activity in erythrocytes in the low micromolar range (28, 40). These studies demonstrated that various bile acids can inhibit ABCC1 transporter activity in a cellular system. In these studies, the researchers monitored the efflux of a pre-loaded fluorescent molecule from erythrocytes. These studies do provide insight into the effects of bile acids on ABCC1 transporter activity, since erythrocytes are known to express appreciable levels of ABCC1. However, it is not the only ABC transporter present in erythrocytes (29, 40–42). In addition to ABCC1, erythrocytes also express ABCC4 and ABCG2 (41, 42).

As we have demonstrated here, the potency of the bile acids appears to be lower in terms of their ability to affect ABCC1-mediated doxorubicin efflux. Where prior studies in erythrocytes have suggested that deoxycholic acid has an IC₅₀ of 16 μ M for inhibition of fluorescent substrate efflux, we have observed IC₅₀'s ~10-fold higher for inhibition of ABCC1-mediated doxorubicin efflux from tumour cells. Unlike the previous studies in erythrocytes, our study focussed on nucleated cancer cells which, as seen in many tumour cell lines, are polyploid. Chromosomal duplications could contribute to substantially higher levels of ABC transporter expression in drug-resistant cells, well above those observed in normal erythrocytes. Indeed, a chromosomal region harbouring the *ABCC1* gene (16p13.1) is amplified almost 100-fold in a multidrug-resistant lung cancer cell line (16). Also, we focussed our study on the effect of bile

acids on the ABCC1-mediated transport of doxorubicin, a clinically relevant chemotherapy agent (43–45). Moreover, we chose to study highly chemoresistant tumour cells, rather than erythrocytes. Nevertheless, the studies in erythrocytes underscore additional effects that bile acids can be expected to exert on normal cell populations in the host. Moreover, our study does not represent the first study on the effects of bile acids on tumour cells. Several groups have attempted to use bile acids and their derivatives as carrier agents for chemotherapy delivery, or even to induce apoptosis (46–48). However, our observations clearly show that the bile acids in this case are not simply acting as carriers for doxorubicin, since no increase in doxorubicin accumulation was observed in tumour cells not expressing ABCC1 transporters.

Our findings related to bile acids provide significant insight into the development of new chemo-sensitizing agents. Furthermore, β -cholanic acid is a known inhibitor of several members of the aldo-keto reductase family, most notably AKR1C3 and AKR1B10, which are known to promote drug resistance through the hydroxylation of doxorubicin to the considerably less cytotoxic doxorubicinol (30).

Clinically, high levels of bile acids are well tolerated in patients (19), suggesting minimal negative systemic effects on host liver and brain tissues. The blood brain barrier is rich in its expression of ABCB1 (8), but unlike MK571, β -cholanic acid would not be expected to inhibit this drug transporter. By inhibiting ABCC1 specifically, β -cholanic acid would be expected to augment accumulation of doxorubicin in ABCC1-expressing tumours, while retaining ABCB1's ability to protect sensitive brain tissues to the damaging effects of doxorubicin.

Despite these possible positive outcome from using bile acids to inhibit ABCC1 activity in chemoresistant cells, the concentrations required to inhibit ABCC1 activity are 10-fold higher than that required for erythrocytes. While normal circulating levels of bile acids in humans are

typically very low (3-8 μ M), it is unclear what effect high concentrations of bile acids would have on the host (21). Higher levels of intracellular bile acids can be detected in certain disease states. However, these conditions usually result in apoptosis occurring in affected cells. In contrast, high doses of another bile acid (ursodeoxycholic acid, derived from bear gallbladders) are usually well tolerated in patients (19).

While we observed no appreciable loss of cell viability in the presence of high levels of bile acids, Mrowczynska et al. showed that erythrocytes could tolerate very high levels of bile acids, much higher than those used in our study. Nevertheless, much work remains to be conducted to better understand the systemic effects and clinical toxicities associated with bile acid treatment. Our study also indicates that the concentration required to inhibit ABCC1 transport is highly dependent on the model used for study and possibly the level of expression of ABCC1. Further experimentation in animal models would be required to examine the therapeutic efficacy and systemic toxicities of doxorubicin in the presence of various bile acids.

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Competing financial interests

There are no competing financial interests

Chapter 8

Overarching Discussion and Future Directions

8.1: Discussion

The phenomenon of chemotherapy resistance is a complex problem that faces physicians in the treatment of cancer (1). As a model for studying chemotherapy resistance, cancer cell lines have proven to be very useful tool. Thanks to the diligent work of a variety of researchers, we have learned about the various mechanisms associated with chemotherapy resistance (2–6). These resistance mechanisms can be observed at several different levels. The first is the entry of the chemotherapy agent into the cells. This can be modulated by a variety of mechanisms, from various solute carriers which can bind to and transport the agents into the cell, to ABC transporters which will actively remove the chemotherapy agents from the cell (6, 7). Mechanisms of resistance can also act in the cytosol of the cells by either metabolizing the chemotherapy agents, sequestering the compounds in lysosomes, or modifying the target to reduce their affinity for the chemotherapy agent (7). A complicating factor of studying these mechanisms is that in most cases, selection of cells for resistance to chemotherapy, as done in laboratory settings, results in activation of several different mechanisms of resistance. While some of these mechanisms appear to develop regularly across many cancer types, others are more difficult to detect. Here we illustrate how the effects of one chemotherapy resistance mechanism can overshadow the role of another. Additionally we demonstrate that a single compound can have effects on two distinct chemotherapy resistance mechanisms.

We began our investigation of resistance mechanisms by examining the role of Aldo-keto reductases. While the cell model we used did express increased levels of a drug transporter, ABCC1, we were interested in examining alternate mechanism of resistance as early studies on the MCF-7_{DOX2-12} cell line did not respond to the then suspected pan-ABC transport inhibitor cyclosporine A. Following microarray analysis of the cell MCF-7 cell line, it was discovered that

members of the AKR superfamily of enzymes showed significant increases of expression. These enzymes, specifically AKR1C3 and AKR1B10, are known to metabolize anthracyclines into less toxic forms (8, 9). An additional characteristic of these enzymes is their role in steroid metabolism, specifically estrogen metabolism. The MCF-7 cell line as a model is characterized as an ER positive breast cancer and as such could be impacted by increases in estrogen synthesis. Given the fact that each of these enzymes is capable of metabolizing doxorubicin to the less toxic doxorubicinol as well as contribute to pro-growth/pro-survival estrogen signalling (10, 11), the observation of increased expression of AKR enzymes would be expected to contribute to chemotherapy resistance. Upon investigation we were able to confirm that the increased expression of AKR1C3 was related to increased levels of estrogen synthesis. This increase in estrogen synthesis was observed and did correlate with increased resistance. However further investigation into the estrogen signalling pathway revealed that estrogen receptor α levels were downregulated in resistant cells which correlated with reduced estrogen signalling. Further studies on the estrogen signalling pathway was the focus of a previous thesis (Chewchuk, 2010). Briefly, estrogen signalling was seen to be impaired in MCF-7_{DOX2-12} cells and shows an inverse relationship to AKR1C3 expression. We propose that the role of reduced estrogen signalling would result in reduced growth rate of cells which would allow for cellular repair following insult by chemotherapy agents. Indeed reduced growth rates are observed in these doxorubicin resistant cells.

The role of AKR1C3 was further solidified by use of a chemical inhibitor, β -cholanic acid. This bile acid binds the active site of the enzyme competitively and prevents the conversion of estrone into estradiol, the final step in estrogen synthesis. Similarly, β -cholanic acid interferes with the binding of doxorubicin to the active site preventing its metabolism to doxorubicinol.

Initial experiments showed that treating MCF-7_{DOX2-12} cells with the inhibitor resulted in a significant restoration of sensitivity to doxorubicin. The effects of the inhibitor were verified by measuring the cells' ability to convert estrone to estradiol. However, follow-up experiments in which AKR1C3 was either over-expressed in wild type MCF-7 cells or knocked down by siRNA in MCF-7_{DOX2-12} cells showed no change in chemo-sensitivity. This finding is in direct contrast to other studies in which AKR1C3 overexpression resulted in increased resistance to doxorubicin (2). We answer this by recognizing that our system was limited in the degree to which we could over express AKR1C3. In the competing models, overexpression of 200 fold were achieved while we were only able to overexpress AKR1C3 to a maximum of 7 fold above wild type levels.

As for the lack of change in cells where we knock down AKR1C3, we observed that the resistant cells also overexpressed AKR1B10 which is also known to metabolize doxorubicin, and would also be sensitive to inhibition by β -cholanic acid. We therefore attempted to knock down both AKR proteins simultaneously in the MCF-7_{DOX2-12} cells. This also resulted in no change in chemotherapy resistance. As a consequence we concluded that while the AKR proteins can contribute to the resistance phenotype, they were not primarily or solely responsible for doxorubicin resistance. We hypothesized, then, that an alternate mechanism had to be responsible for the cells' ability to survive doxorubicin. Upon re-examination of the micro-array data, as well as previous studies on the MCF-7_{DOX2-12} cell line, we noted the overexpression of ABCC1, a transporter protein known to export anthracyclines from the cell to the extracellular space or sequester them in lysosomes. Additionally, experiments designed to measure the amount of doxorubicin retained within cells illustrated that upon treatment with β -cholanic acid, MCF-7_{DOX2-12} cells retained increased levels of doxorubicin relative to their untreated controls.

This lead to the hypothesis that β -cholanic acid could be having an impact on resistance mechanisms other than AKR1C3.

As discussed in chapter 7 of this thesis, we tested the possibility that β -cholanic acid had the ability to block the function of the ABCC1 transporter. We found that β -cholanic acid had profound effects on doxorubicin sensitivity in cell lines which expressed ABCC1. At this point in the study, a question remained as to the mechanism of inhibition. One possible way that bile acids could impact on ABCC1 function would be to serve in their most basic role by solubilizing lipids and cholesterol molecules in the cell membrane and altering the fluidity of the membrane which could disrupt the 3D structure of the ABC transporter. While membrane fluidity is a factor that should be considered when studying trans-membrane proteins, it is an unlikely factor in this situation, as we would expect to see the same effect in cells expressing ABCB1 as a transporter. However, upon testing β -cholanic acid on MCF-7_{TXT} and A2780_{ADR} cells, we saw no effects on restoring doxorubicin retention. This is an important finding since the MCF-7_{TXT} cell line was selected for resistance to paclitaxel and is known to express high levels of ABCB1 (3). While this cell line shared a common history with the MCF-7_{DOX2-12} cell line, it was selected for resistance to an alternate chemotherapy agent, and as such may not be sufficiently analogous for comparison. To verify that the effect we observed was not dependent on the drug used for selection for resistance we also tested the A2780_{ADR} cell line, an ovarian cancer cell line which was selected for resistance to doxorubicin and known to express ABCB1 as a mechanism of chemotherapy resistance (12). Once again, no effect on drug retention was observed. We suspected that the effect of β -cholanic acid on chemotherapy retention within the cell was specific to the ABCC1 transporter as we confirmed that and ABCB1 inhibitor was able to resort doxorubicin retention in these cells.

One of the characteristics of ABCC1 is that it possesses 3 distinct substrate binding sites, each of which is capable of recognizing different substrates (13,14). β -cholanic acid has a similar structure to doxorubicin and may bind to the same substrate binding site as doxorubicin, as it does in the AKR enzymes. This would prevent doxorubicin binding the site and subsequently prevent its efflux from the cell. An alternate possibility is that β -cholanic acid is interacting with one of the other substrate binding sites preventing the normal function of the transporter. In either case, an ideal system to test these possibilities would be to first use in silico analysis and attempt to map the structure of β -cholanic acid onto the substrate binding sites of ABCC1. This would require a 3D crystal structure of the protein, which unfortunately does not exist due to the complex nature of ABCC1.

The data collected thus far suggests that in addition to the effects of β -cholanic acid by inhibiting AKR enzymes, it is also capable of interfering with the activity of ABCC1. The exact mechanism by which β -cholanic acid interferes with ABCC1 is yet to be elucidated, however preliminary findings suggest a possibility of non-competitive inhibition (see appendix **Figure 9-1**). It should be noted that the data pertaining to the exact mechanism of inhibition was acquired from a whole cell system. This method of observing kinetics of membrane transport present several problems. First is the issue of membrane composition. The plasma membrane is comprised of many different lipid compounds each contributing to different structures of the membrane. These include but are not limited to lipid rafts, which may play a role in protein transporter structure/function, cholesterol and other derivatives, which play a role in membrane fluidity and can affect the rate at which solutes cross the plasma membrane. Any of these membrane structures can be disrupted by bile acids which could account for variance in the changes in drug uptake in the cells. We also note that the changes in K_m and V_{max} , while

statistically different, are much smaller than we expected based on the total drug uptake measured by fluorescence.

The benefit of using whole cells in a kinetic assay is that the membrane will contain other proteins and sugars which would support the cellular structure and allow the system to better tolerate the bile acids being tested. However this system does add complications to measuring the function of a single resistance factor. Knowing that β -cholanic acid can impact on enzymatic processes within the cells, namely the AKR metabolic enzymes, attempting to study the effects on ABCC1 transport is difficult. To minimize the variables associated with whole cell systems we used a stable transfected cell model to study the effects of β -cholanic acid on ABCC1 transport.

Due to the nature of ABC transporters, it is impossible to study their function in the absence of a plasma membrane. In addition, bile acids are commonly used to dissolve simple lipids. Deoxycholic acid, for instance, is a common additive in cell lysis reagents to facilitate emulsification of the phospholipid bilayer of the cells. Note that this is at higher concentrations than were used in our experiments. Due to the possible complications associated with use of bile acids we opted to use a whole cell system to analyze the kinetics of transport inhibition. The alternative, and more commonly used method, involves the use of specially designed micelles which contain the transporter along their surface. These micelles would then be treated with ATP and the necessary substrates. For study of ABCC1 transport the typical substrates would be methotrexate and glutathione. One could then add the inhibitors to the liposomal mix and measure the amount of methotrexate which enters the liposome. The concern using such a system is that the bile acids may completely disrupt the micelles preventing any usable data from

being gathered. Nevertheless this system could be employed for future experimentation and attempt to better elucidate the nature of inhibition on ABCC1 by β -cholanic acid.

While β -cholanic acid is commonly used to treat liver disease, this is typically accomplished by oral ingestion. As noted previously, β -cholanic acid as a treatment for liver disease promotes the secretion of secondary bile acids from the liver which are normally toxic in high quantities. Additionally, β -cholanic acid is not typically absorbed in the digestive tract the way cholic or chenodeoxycholic acids are. As a result, we postulate that oral administration of β -cholanic acid would not be effective at treating most cancers. Furthermore since bile acids are known to promote cell growth and survival through activation of the farnesoid x receptor, β -cholanic acid is not likely to be a good candidate in treating cancers of the digestive tract such as colon cancer. We would propose that β -cholanic acid would rather be a useful tool for treating cancers which are shown to express ABCC1 and/or AKR1C3 or 1B10 as primary mechanisms of chemotherapy resistance. Additionally, for the treatment to be effective it would have to be administered intravenously in parallel with traditional chemotherapy to allow the drugs to better access the cancers.

This brings us to one of the major limitations of β -cholanic acid as a supplemental treatment for chemotherapy resistant cancer, and that is the relatively high concentration needed to inhibit each AKR1C3 and ABCC1 (100-200 μ M). We note that these concentrations are very close to the maximum levels of β -cholanic acid which can be dissolved in aqueous solutions. This could present problems in administering β -cholanic acid intravenously as it could potentially precipitate in the blood stream during treatment. This could lead to damaging emboli which could be lethal to the patient. In addition, we noted in an earlier chapter that while low dose of circulating bile acids could have beneficial effects on cell survival, high concentrations

of bile acids are associated with tissue damage (liver fibrosis as an example). Additionally, while β -cholanic acid is useful at removing bile acids in liver disease, it could have a detrimental effect when administered intravenously by promoting the secretion of cholic acid and chenodeoxycholic acid into the circulatory system. For these reasons we propose that, rather than use β -cholanic acid directly for co-treating chemotherapy resistant cancers, artificial derivatives would be more useful for patient care.

8.2: Future Directions

As we see in a variety of systems *in vitro*, chemotherapy resistance is not restricted to any one mechanism, although one mechanism will tend to dominate over others. Typically, as cells develop resistance to chemotherapy they will adopt multiple mechanisms to promote their survival, and these mechanisms may change during their selection for resistance as the selective pressures change. As we discussed earlier, cells can alter many aspects of their physiology to combat the damaging effects of chemotherapy. Here we studied the roles of 2 major contributors to anthracycline resistance, ABCC1 transporters and AKR1C3 (doxorubicin metabolizing enzyme). While these two modes of resistance are distinct, they can each function to protect cancer cells from the damaging effects of doxorubicin. As we have demonstrated however a single compound, β -cholanic acid, is capable of inhibiting each of these mechanisms of resistance. By simultaneously blocking chemotherapy export and metabolism, we could prevent the cancer cells from detoxifying themselves. The added benefits of this are that, by blocking AKR metabolism of doxorubicin, many of the toxic side effects caused by doxorubicin metabolites could be avoided.

As we have noted, since the optimum method of deliver of β -cholanic acid for the treatment of resistant cancer would be intravenous injection, and normal circulating levels of bile

acids are typically very low, there is a concern that high doses of β -cholanic acid added to the circulation could have toxic side effects. It is difficult to say for certain if this would be the outcome, as bile acids have never been administered in this manner before. It is known that low doses of β -cholanic acid administered orally can have regenerative effects on liver tissue, however it is not known what effects these doses would have if applied directly to the circulation. Injecting β -cholanic acid intravenously could have protective effects on various tissues of the body or detrimental ones. An important step in developing β -cholanic acid as a treatment for doxorubicin resistant cancers would be to test the tolerances of escalating doses of β -cholanic acid in animal models. Following this, we could use various doses of β -cholanic acid in conjunction with doxorubicin to treat animals with xenografted ABCC1 expressing tumours to determine if this method could be successfully applied to *in vivo* systems.

While β -cholanic acid is a promising lead in the treatment of ABCC1 and AKR expressing chemotherapy resistant cancers, we have outlined some of the possible complications due to the high concentrations needed. We would therefore propose that the chemical structure of β -cholanic acid could serve as a template for design of new compounds which would target ABCC1 and AKR1C3 as a future direction. The goal of this would be to produce a compound which would be effective at blocking both resistance mechanisms while minimizing potential side effects. This strategic design would present a major challenge. With β -cholanic acid exhibiting a high level of specificity for interfering with ABCC1 and not ABCB1, β -cholanic acid treatment would likely not have the same negative side effects as other ABCC1 inhibitors like MK571 which can have off-target effects on ABCB1 at higher concentrations. Among these are neurological effects suffered by patients when ABCB1 is inhibited due to the loss of protection at the blood-brain barrier (15). We propose therefore that β -cholanic acid would fill

the role of a more targeted agent against ABCC1 as a resistance mechanism could therefore avoid some of the side-effects of less specific agents. These new agents would first be tested for their efficacy *in vitro* against both modes of resistance, as well as specificity to ABCC1 and AKR1C3. Following this, as with β -cholanic acid, tolerance and efficacy in animal models would have to be tested before any attempt can be made clinically. If successful, development of a specific and potent inhibitor of ABCC1 and AKR1C3 could prove extremely useful in patient care by providing new treatment options for difficult cancer cases.

8.3: Concluding Remarks

While chemotherapy resistance continues to be a growing issue with treatment of various cancers. We present here a new tool that can potentially be used to combat some of these resistant cancers. The use of bile acids in medical treatment is not without precedent, admittedly not for cancer treatment. While much work would need to be performed to validate the observation here as well as optimize the treatment condition for *in vivo* systems, we believe that the use of bile acids (specifically β -cholanic acid) or their chemical derivatives would greatly improve survival outcomes as well as improve quality of life. This would be achieved in a two-fold process by sensitizing cancers to conventional therapies and minimizing toxic side effects from chemotherapy metabolites. Furthermore by specifically targeting only AKR1C3 and ABCC1, we could avoid off target effects commonly seen in current anti-resistance treatments.

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Chapter 9

Appendix

9.1: Expanded Materials/Methods

9.1.1: Cell Culture

The MCF-7 (breast cancer) cell line was purchased from ATCC (Manassas, VA), maintained in high glucose DMEM supplemented with 10% FBS and 100 µg/ml streptomycin and 100 units/ml penicillin (all from Hyclone, Mississauga, Ontario). Cells were subcultured once every 7-8 days, or once cells had reached 90% confluence, and routinely supplemented with fresh media every 4 days. Cells were maintained at 37° C in 5% CO₂. For sub-culturing, cells were washed once with sterile PBS followed by the addition of 3 ml (for T75 flasks (Sarstedt)) of sterile Trypsin 0.25% EDTA solution (Gibco). Once the cells were lifted from the growth surface, the cell suspension was then added to 1 ml of growth medium to inactivate the trypsin, and the flask was washed again with sterile PBS to collect any residual cells. The cell suspension was subjected to centrifugation at 233 x g for 10 min at 20° C in a Beckman Coulter Allegra X-12R centrifuge. Cell pellets were resuspended in media and re-plated. Cells were previously selected for resistance to either Doxorubicin (dox) or Epirubicin (epi) by treating cells with an initial concentration of drug at 1,000-fold below the IC₅₀. Drug concentrations were gradually increased in either 3 fold or 1.5 fold increments depending on the cells' ability to tolerate the new drug concentration. A co-culture control cell line was selected in parallel which had no drug added. Drug resistant cells were maintained in media supplemented with either dox or epi. Drug concentrations were as follows: Dox₁ – 3x10⁻⁷ M, Dox₂ Dose 7 – 6.48x10⁻⁹ M, Dose 8 – 1.94x10⁻⁸ M, Dose 9 – 2.91x10⁻⁸ M, Dose 10 – 4.36x10⁻⁸ M, Dose 11- 6.54x10⁻⁸ M, Dose 12 – 9.81x10⁻⁸ M, Epi Dose 12 – 8.52x10⁻⁷ M. Resistant cells were removed from drug-containing media 3-4 days prior to being subjected to experimentation. For experiments involving estrogen signalling and/or metabolism, Dextran/Charcoal (DC) filtered media was used.

9.1.2: Dextran Charcoal Filtering

DMEM medium supplemented with 10% FBS and antibiotics was treated with 0.5% (w/v) dextran coated charcoal (Sigma, St. Louis, MO), and heated to 50 °C with constant shaking for 30 min. The charcoal stripped media was then sterile filtered (Sarstedt 0.22 µm, 500 ml). DMEM media was supplemented with 10^{-7} M estradiol for ER signalling experiments, or 10^{-7} M estrone (both from Sigma, St. Louis, MO) for metabolic testing.

9.1.3: Trypan Blue Exclusion Assay

Cells were grown in the absence of chemotherapy agent for 1 week prior to plating. Cells were washed, lifted and counted, plated in 10 cm plates (1,000,000 cells/plate), and left to incubate for 9 days. Individual plates were harvested every 2 days beginning with 24 h post plating. Cells were counted on a hemocytometer using the Trypan Blue Exclusion stain (Fisher). Briefly, cells were suspended in 1-10 ml of media, and a 15 µl aliquot of each cell suspension was stained with an equal volume of 1% Trypan Blue. Total cell number was determined by counting the sum of Trypan-excluding cells and Trypan-stained cells. This protocol was modified to a 6 well format by plating 100,000 cells/well and resuspending the cells in 100 to 1,000 µl of media. Growth rate was calculated from a Boltzmann Sigmoidal Curve (Graphpad Prism V 5.0).

9.1.4: Estrone Metabolism Assay

5×10^5 cells/well were plated in 6 well plates (Sarstedt), in DMEM medium supplemented with 10% FBS and antibiotics and allowed to adhere overnight. The next day, cells were washed and 5 ml of DC-DMEM was added to each well. Cells were either treated with vehicle control (5% DMSO), 10^{-7} M estrone, 10^{-7} M estrone with 5 nM letrozole, or 10^{-7} M estrone with 0.2 mM

β -cholanolic acid. Cells were treated for 24 h at which point aliquots of media were collected and stored at -20 °C. Samples were diluted 10-fold prior to being tested for estradiol levels. Estradiol levels were assayed using E2 ELISA kits (US Biological) as per the manufacturer's protocol. Briefly, 50 μ l of standards and unknown samples were added to individual wells, coated with an antibody specific for E2, in duplicate. In addition a competitive HRP conjugated estradiol molecule was added to each sample well. This substrate competes with the unconjugated E2 substrate in the samples and will result in a signal developed that correlated to the E2 concentration in each well. Samples were incubated at room temperature for 1 h on a Stoval Belly Dancer plate shaker with mild shaking to facilitate even dispersal of the binding substrates. After the incubation period, the samples were decanted and were washed 3 times with provided wash buffer. Colorimetric reagent was next added to each well and incubated for 1 h at room temperature. The plates were read on a Spectramax 340 PC spectrophotometer at a wavelength of 650 nm. Concentrations were extrapolated from a standard curve based on percent absorbance from the negative control. All data was analyzed using Graphpad Prism software (version 5.0 Graphpad Prism Software Inc.).

9.1.5: Clonogenic Assay

Cell survival was measured using a clonogenic assay. Cells were first collected by trypsinization, stained with Trypan blue, and counted as previously described (section 2.3). Cells were then diluted to a concentration of 4,000 cells/ml, and 5 ml of this cell suspension was added to T25 flasks (i.e., 200,000 cells/flask) (Sarstedt). 18-24 h post plating, the culture medium was removed and replaced with 5 ml of medium supplemented with a 3 fold increasing concentration of drug. The drug concentration ranged from 3×10^{-11} M to a maximum concentration of 3×10^{-6} M, with one flask left untreated as a negative standard. The cells were incubated as described

previously, for 24 h. Following the 24 hour incubation period, cells were lifted as previously described and centrifuged with collected media and wash buffers to ensure that all viable cells were retained. The solutions were decanted and then the cell pellets were resuspended in 300 μ l of full media and then transferred to 13 ml tubes containing 2.6% methylcellulose (Shin Etsu, Biddle Sawyer, NY.) supplemented with 30% FBS (Gibco). Methylcellulose/cell suspensions were thoroughly vortexed to ensure even cell distribution, and then aliquoted into 6 well clustered dishes (Corning). Colonies were counted 13-14 days post-plating.

9.1.6: Plasmid DNA Transfection and Cloning

Plasmids containing the open reading frame for AKR1C3 used for overexpression, were ordered from Invitrogen. ORF pENTR211 plasmids (see appendix) were received transformed into bacterial vectors (DH5- α). Bacteria were cultured on LB-Agar plates containing 50 μ g/ml of kanamycin as a selective agent. The ORF was cloned from the host plasmid by linearizing the plasmid with EcoRI restriction enzyme followed by a PCR reaction to amplify the ORF only. PCR mix contained 0.2 mM dNTP mix, 1.5 mM MgCl₂, 0.2 μ M each of forward and reverse primers (F: 5'-GCTAAGATCTTCATGGATTCCAAACACCAGTGTG-3', R: 5'-TCGACTCGAGGTACAAGAAAGCTGGGTTCTAATATTCATC-3'), 2.5 units of Qiagen HotstarTaq, and 14.7 ng of template DNA. The ORF underwent 25 cycles in a Techne TC 312 thermocycler.

The PCR products were run on a 0.9 % agarose electrophoresis gel to isolate the ORF product. Using a transilluminating UV table, the single band was removed from the gel and the Gel/ORF was mixed with 3 volumes of QG buffer (Qiagen) and incubated at 50 °C for 10 min (until gel completely dissolved) according to the manufacturer's protocol. One volume of isopropanol was added to the tube and mixed. The solution was added to a QIAquick column and

subjected to centrifugation for 1 min at 17,000 x g. The column was washed with PE buffer and once again spun for 1 min at 17,000 x g. The ORF DNA was eluted using 50 µl of buffer EB and once again subjecting the column to centrifugation for 1 min at 170 00 x g.

The AKR1C3 cDNA was next ligated into a pCMV-Tag plasmid such that the FLAG-Tag was incorporated into the c-terminal domain. The ligated plasmid was next cultured on kanamycin-containing plates to select for AKR-Tag plasmid expression. The bacterial DNA was purified using Qiagen QIAprep miniprep plasmid extraction kits and QIAprep maxiprep kits (Qiagen). Plasmid concentration and purity were determined using an A260/280 ratio measured on a Beckman Coulter U530 spectrophotometer. The AKR-Tag insert was confirmed by sequencing (MOBIX) and by restriction endonuclease analysis.

Stable clones were next generated by transfecting MCF-7_{CC} cells grown to 90% confluence in 10 cm plate with 24 µg of plasmid DNA complexed to 24 µl of Lipofectamine 2000 (Invitrogen). Complexes were prepared by incubating 24 µg of plasmid DNA and 24 µl of Lipofectamine, each, in 1.5 ml of Optimum medium (Invitrogen) for 5 min. Following this incubation period, the two volumes of optimum were combined to generate the DNA-Lipofectamine complexes. These complexes were allowed to form for 25 min prior to being added to 10 cm plates containing 15 ml of antibiotic free DMEM supplemented with 10% FBS. Cells were transfected overnight, and then subjected to selective screening using 2.0 mg/ml G418 (Sigma, St. Louis, MO). Selective concentration was determined by generating a kill curve by treating untransfected cells with increasing concentrations of G418 (0-2.5 mg/ml). The concentration was chosen based on the lowest concentration to kill over 80% of the cell population after 1 week of treatment (see appendix). Selective pressure was maintained for 72 h post-transfection. A population of cells were subcultured and frozen for storage in liquid

nitrogen, while individual cell colonies were also selected, grown, and frozen for later use. Empty vector clones were generated as a negative control using the same protocol described above. A total of 46 AKR-Tag clones and 24 empty vector clones were collected and stored.

Transient AKR1C3 over expressing cells were also generated in the same manner as the stable clones with the following exceptions: 24 µg of plasmid DNA was used complexed to 60 µl of Lipofectamine 2000. The cells were transfected for 18 h and were then replated for subsequent experiments. The amount of overexpression was verified via Western Blot for each transiently transfected experiment.

9.1.7: Protein Extraction

MCF-7 co-culture control or drug resistant cells were grown to 70-80% confluence on 10 cm plates (Sarstedt) in DMEM medium supplemented with 10% FBS for basal protein extraction, or switched to DC-DMEM 24 h prior to testing for estrogen signalling treatments. For estrogen-treated cells, the cells were treated with either a 5% DMSO vehicle control or 10^{-7} M Estradiol for 30 min for optimum phosphorylation of estrogen receptor. The culture medium was removed and the cells were washed 2-3 times with PBS (0.15 M NaCl, 2.7 mM KCl, 1.0 mM KH_2PO_4 , 10 mM Na_2HPO_4). Cells were lysed using 500 µl of RIPA buffer (1% (v/v) NP-40, 0.5% (w/v) deoxycholic acid, 0.1% (w/v) SDS, dissolved in PBS) supplemented with 1x Complete Inhibitor Cocktail (Roche), 10 µM NaF, 2 mM Na_3VO_4 and 1 µM PMSF (all from Sigma, St. Louis, MO). Cells were lifted from plates using plate scrapers, and the crude lysates were left on ice for no more than 5 min before being sheared by being passed through a 21-gauge needle 5-10 times. Crude lysates were then subjected to centrifugation at 17,000 x g for 10 min. at 4 °C. The supernatants were collected and stored at -80 °C. 40 µl aliquots were taken for total protein quantification using BCA assay, and quantified according to the manufacturer's protocol

(Pierce). Briefly, extracts and standards were added into the bottom of 96 well assay plates. BCA reagent was added, and plates were incubated at 37 °C for 30 min. The protein samples were read on a Spectramax 340 PC spectrophotometer at a wavelength of 540 nm. The data were analyzed and concentrations were extrapolated using Graphpad Prism software (version 5.0, Graphpad Inc.). Protein samples were then aliquoted into 1.5 ml tubes containing 1x Laemmli sample buffer (0.05 M Tris (pH 6.8), 8% (v/v) glycerol, 0.03 M DTT, 0.08% (w/v) bromophenol blue) and boiled for 5 min prior to being resolved on gels by electrophoresis.

9.1.8: Western Blot

9.1.8.1: SDS PAGE Gel Electrophoresis

Protein extracts were run on 10% or 12% (for resolving smaller proteins) polyacrylamide gels using the Tetra-gel system (Bio-Rad) in a 1x running buffer (25mM Tris, 192 mM Glycine, 3.5 mM SDS). Precession Pro Standard ladder (Bio-Rad) was used for protein size comparison. Samples and ladder were subjected to an initial charge of 90 V for the duration of 10-15 min, followed by a charge of 130 V for 30-45 minutes depending on resolution of band sizes. Proteins were next transferred to nitrocellulose membranes using a Bio-Rad semi-dry transfer apparatus in transfer buffer (48 mM Tris, 39 mM Glycine, 0.037% (w/v) SDS, 20% (v/v) Methanol). Proteins were using 12 V for 45 min. Successful transfer of the proteins was confirmed through staining with 1% (w/v) PonceauS stain (Sigma, St. Louis, MO). Membranes were blocked using either a 5% (w/v) skim milk solution or a 5% (w/v) BSA solution suspended in 0.1% TBST (20 mM Tris pH 8.0, 137 mM NaCl, 0.1% (v/v) Tween 20) (in the case of probing for phosphorylated proteins) for 1 h at room temperature.

9.1.8.2: Blotting

Membranes were incubated overnight at 4 °C while being probed with primary antibodies (concentrations varied based on probe of interest, averaged dilution was 1:1,000 from commercial stock). Primary antibody was diluted in either 5% (w/v) skim milk solution or 5% (w/v) BSA solution and membranes were incubated on a shaker at 4 °C, overnight. After the incubation period, the membranes were washed 3 times in 5 min intervals with TBST (20 mM Tris pH 8.0, 137 mM NaCl, 0.1% v/v tween-20). Secondary antibodies were applied, in a 1:10,000 dilution from commercial stock suspended in a 5% skim milk solution, for 1 h at room temperature with gentle shaking. Membranes were next washed 3 times with TBST and once with TBS (20 mM Tris pH 8.0, 137 mM NaCl) in 5 min intervals. Chemiluminescent reagent (West Pico, Pierce) was added next and the membranes were incubated for 5 min. Visualization of the chemiluminescent reactions were observed by either exposure to film followed by development, or via gel doc camera system (FluorChem™, A Innotech) depending on intensity of signal. Integrated density values were acquired using AEase FC software and analyzed using Microsoft Excel (Microsoft Office 2007, Microsoft).

Table 9-1 Antibody Dilutions

<i>Material</i>	<i>Source</i>	<i>Titre Used/Application</i>	<i>Company</i>
Anti-Rabbit-HRP conjugate	Goat polyclonal	1:10 000 WB	Santa Cruz
Anti-Mouse-HRP conjugate	Goat polyclonal	1:10 000 WB	Santa Cruz
Anti-GAPDH	Mouse monoclonal	1:10 000 WB	Santa Cruz
Anti-ERα-P-Ser118	Mouse monoclonal	1:1 000 WB	Cell Signal
Anti-ERα	Mouse monoclonal	1:1 000 WB	Cell Signal
Anti-AKR1C3	Mouse monoclonal	1:1 000 WB	Sigma
Anti-ERK-P- T202/Y204	Rabbit monoclonal	1:1 000 WB	Cell Signal
Anti-BCL-2	Mouse monoclonal	1:200 WB	Santa Cruz
Anti-FLAG	Mouse monoclonal	1:1 000 WB	Stratagene
Anti-Cyclin D1	Mouse monoclonal	1:1 000 WB	Upstate
Anti-γ-Tubulin	Monoclonal mouse	1:20 000 WB	Sigma
Anti-γ-Tubulin	Polyclonal rabbit	1:10 000 WB	Sigma
Anti-ERK	Rabbit monoclonal	1:1 000 WB	Cell Signal
Anti-ERβ	Rabbit polyclonal	1:1 000 WB	Santa Cruz
Anti-ABCC1	Mouse monoclonal	1:1000 WB	Cell Signal

9.1.8.3: Stripping and Re-Probing

When probing for phosphorylated proteins, it was necessary to strip the phospho-specific antibodies and re-probe the blots with a non-phospho-specific antibody. Membranes were incubated for 1 h at 50 °C in stripping buffer (6.24 mM Tris pH 6.7, 2% (w/v) SDS, 100 mM β -mercaptoethanol). The membranes were washed twice in TBST for 5 min, each followed by a brief wash in TBS. A test exposure was conducted by adding ECL to the membranes and exposing the membranes to fresh film for 10 min to confirm the antibodies had been stripped. Membranes were then re-blocked using 5% (w/v) skim milk in TBST for 1 hour. Following blocking, membranes were probed for proteins as, described above.

9.1.9: Q-PCR

9.1.9.1: RNA extraction

Cells were grown in 10 cm plates with DMEM supplemented with 10% FBS and grown to 80% confluence. Qiagen RNeasy kits were used to extract total RNA from the cells. Briefly, the cells were washed twice with PBS, and 600 µl of buffer RLT (supplemented with 10 µl of β-mercaptoethanol per ml) was added to each plate. Cells were then scraped and homogenized by shearing through a 20 gauge needle (5 times) and added to an equal volume of 70% ethanol. Samples were applied to an RNeasy spin column and subjected to centrifugation at >8,000 x g for 15 sec. Columns were washed and the RNA was eluted in water. All RNA samples were frozen and stored at -80 °C, in addition 5 µl aliquots were taken for RNA analysis.

9.1.9.2: RNA analysis

All RNA samples were analyzed using RNA 6,000 Nano Assay Lab Chip kit (Agilent) as per manufacturer's directions using an Agilent 2100 Bioanalyzer. Briefly, all assay reagents were warmed to room temperature prior to use, RNA samples were denatured by boiling at 70 °C for 2 min. and 1 µl samples were analyzed for quality as well as quantity. In addition to quantifying the RNA, the Bioanalyzer also provides information regarding the 28S/18S ratio.

9.1.9.3: RT-PCR

RNA (1 µg) was treated with DNase (Invitrogen) prior to reverse transcription to remove any residual DNA. RNA was reverse transcribed by first adding a 1x first strand buffer (Invitrogen), DTT, 10 mM dNTPs, 20 ng/µl T20 primers, and 200 units of M-MLV reverse transcriptase. This mixture was incubated at 37 °C for 2 h and then incubated at 95 °C for 5 min to inactivate the M-MLV. Samples were stored at -80 °C.

9.1.9.4: Q-PCR

cDNA derived from co-culture control dose 12 cells was used to generate a standard curve (0.25-0.004 x dilution). Samples were diluted 0.062 fold from stock in RNase/DNase free water. 5 µl of each standard and unknown sample was loaded in triplicate on a 96 well PCR plate (Corning). Gene specific primers were diluted to 200 µM in RNase/DNase free water and further diluted to a working concentration of 300 nM. 7.5 µl of working primer solution was added to each well containing template cDNA. Finally 12.5 µl of SYBR Green mix (Bio-Rad) is added to each well. The plate was sealed with clear heat resistant adhesive film to prevent evaporation and the plates were subjected to centrifugation at 100 x g for 5 min. Plates were placed in a Q-PCR machine (Applied Biosystems) and run for 40 cycles followed by a dissociation curve analysis. Cycling began with an initial heating of the samples to 95 °C for duration of 10 min to activate the TAQ polymerase. This was immediately followed by further heating the sample at 95 °C for 15 sec followed by cooling and annealing of the primers at 55 °C for 15 sec then elongation of the primers at 72 °C for 30 sec. This pattern was repeated for 40 cycles. Upon completion of the 40 cycles, the samples underwent dissociation analysis which involved heating the samples to 95 °C and gradually cooling them to 60 °C then gradually heating the samples again to 95 °C. Fluorescence measurements were taken every cycle as well as at every degree change for the dissociation analysis. Data was captured using a 7900HT Sequence Data System (Applied Biosystems) using Sequence Data System acquisition software (V. 2.1, Applied Biosystems). Gene expression was standardized to S28 expression.

9.1.10: ER- α TRANS-AM Analysis

Active nuclear ER- α levels were assayed using TRANS-AM kits purchased from Active Motif. These kits function as an ELISA analogue to the gel mobility shift assay. Briefly, nuclear

extracts were prepared by growing cells in 10 cm plates to confluence. The high confluence is required to collect sufficient amounts of nuclear protein for the assay. Nuclear extracts were prepared as per manufacturer's instructions. Protein concentrations were determined using Bradford Assay as the Lysis Buffer (Active Motif) contained DTT which would interfere with the BCA assay.

ER- α transcription factor assay was performed as per manufacturer's directions. Briefly, nuclear protein samples were loaded onto 96 well assay plates coated with Oligonucleotides corresponding to the estrogen response element. These were allowed to incubate at room temperature for 1 hour with mild agitation to facilitate binding of the transcription factors. Following the incubation period, the samples were washed 3 times with 1x Wash Buffer (Active Motif) and the plates were tapped dry using absorbent paper. Primary antibody (anti-ER- α , 1:2000) (Active Motif) was added to each sample well and allowed to incubate at room temperature for 1 hour without agitation. Samples were then washed 3 times with 1x Wash Buffer as previously described. Secondary anti-rabbit-HRP conjugated antibody was then added (1:2000) (Active Motif) to each sample well. Plate was allowed to incubate for 1 hour at room temperature without agitation. Samples were then washed 4 times as previously described. Colorimetric substrate was added to each well and allowed to develop for 5 min at room temperature. Immediately after the developing period, stop solution was added to each well to halt the developing process. Samples were read on a Spectramax 340 PC spectrophotometer at a wavelength of 450 nm. Plates were blanked to sample wells that had no protein added as a negative control. All data was analysed using Graphpad Prism software (V. 5.0, Graphpad Software Inc.)

9.1.11: ER- α Knockdown

ER- α levels were artificially knocked down using Artemisinin. Artemisinin interferes with transcription of the ESR1 gene by blocking transcription factor binding to the promoter region of the gene. Cells were plated at roughly 30% confluence in 10 cm plates containing DMEM supplemented with 10% FBS and antibiotics. 24 h post plating, the media was removed and replaced with DMEM supplemented with 10% FBS and either 300 μ M Artemisinin or 0.1% v/v DMSO as a vehicle control. Cells were allowed to grow at 37 °C, 5% CO₂ for 72 h. Following the treatment period, proteins were extracted from the cells as previously described. Protein samples were assayed via western blot technique for ER- α , BCL-2, and Cyclin D1 levels.

9.1.12: Chemotherapy uptake analysis

9.1.12.1: Fluorescent based uptake analysis

Steady-state doxorubicin retention was measure via flow cytometry. Cells were seeded onto 6 well plates at a density of 200,000 cells per well and allowed to set. Cells were treated with 2 μ M doxorubicin, various concentrations of β -cholanolic acid, deoxycholic acid, MK571, probenecid or a combination of one of these with doxorubicin for a period of 6 h. Cells were collected and suspended in 1 ml PBS. Suspended cells were run on an FC500 flow cytometer (Beckman-Coulter) using an FL2 (575 nm) filter to detect doxorubicin fluorescence. Data shown represents the mean of results obtained in 3 independent experiments and 10,000 events were measured in each individual experiment.

9.1.12.2: Radiolabeled uptake analysis

Steady state docetaxel uptake was measured using ³H tagged docetaxel. Briefly, cells were plated at 200,000 cells per well in a 6 well plate. 18-24 h post plating cells were treated with ³H-docetaxel for 6 h. Cells were washed 3 times with PBS and lifted from the plates using

200 μ L trypsin/EDTA. Lifted cells were added to scintillation tubes. Scintillation fluid was added to each tube and placed into a Beckmann coulter scintillation counter. Scintillation counting was performed to measure the amount of radio labeled docetaxel in each sample.

9.1.12.3: Kinetic uptake analysis

To determine the nature of inhibition of ABCC1 transport activity by β -cholanic acid, (14 C)-doxorubicin accumulation was measured in MCF-7_{DOX2-12} cells at different doxorubicin concentrations in the presence and absence of cholanic acid for 4 hours and Lineweaver-Burke plots of 1/uptake velocity versus 1/doxorubicin concentration were generated. Cells were then collected and cellular doxorubicin levels quantified by liquid scintillation counting.

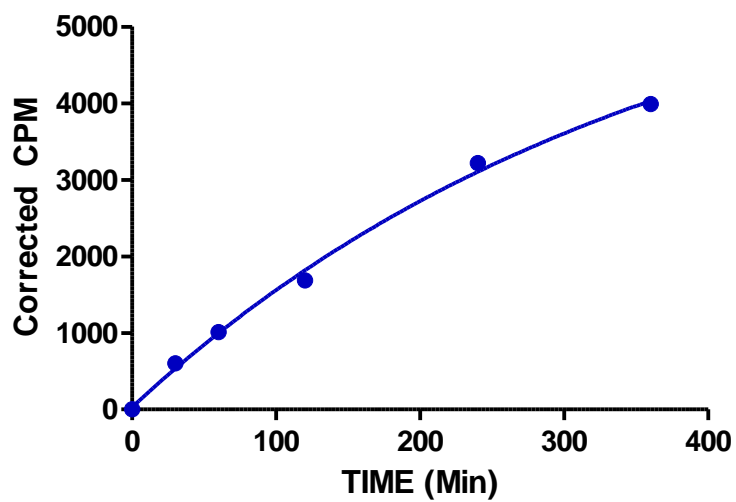
9.1.13: Glutathione retention analysis

Glutathione levels in MCF-7_{DOX2-12} whole cell extracts were measured using a glutathione detection kit from ENZO. Cells were plated in 10 cm plates at a density of 2×10^6 cells per plate. Cells were pre-treated with buthionine sulfoximine (50 μ M for 1h) Cell lysates were collected and glutathione assayed as per kit instructions. Data shown are mean of 3 independent trials \pm SEM.

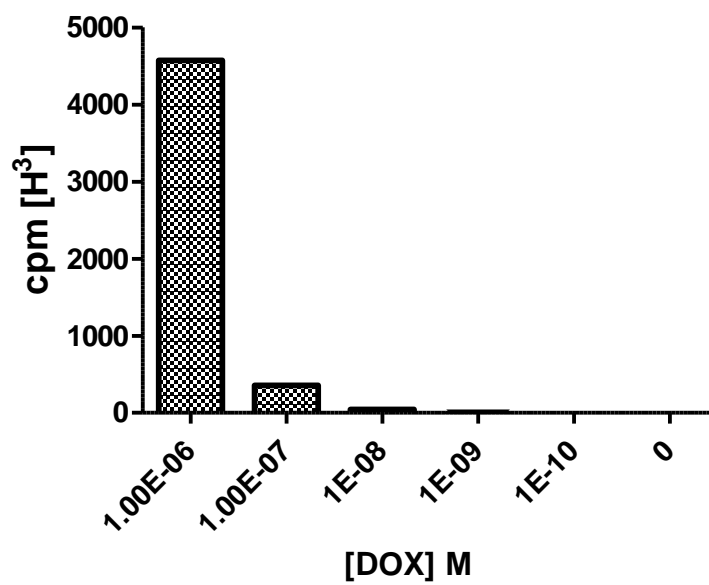
9.2: Supplemental Data

Figure 9-1 Analysis of doxorubicin uptake kinetics.

Hek293 radioactive uptake time course 1uM C¹⁴ Doxorubicin



Measure of Doxorubicin uptake at 4 h



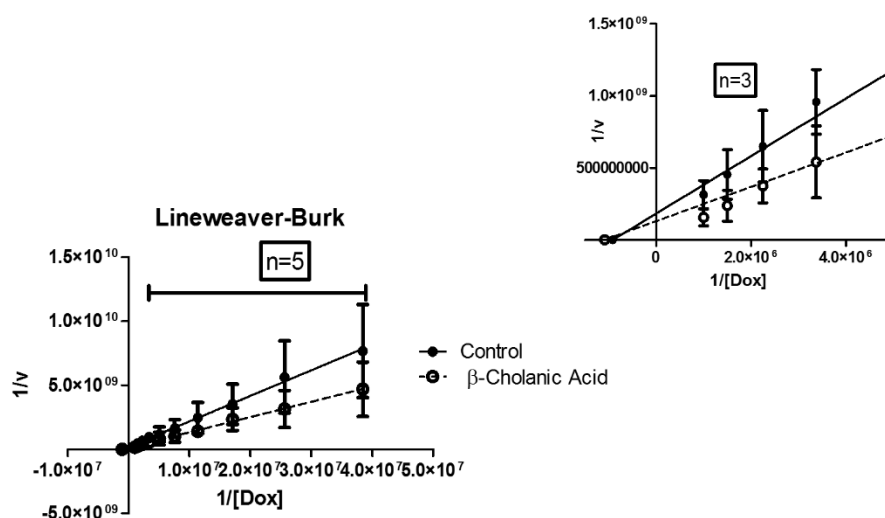


Figure 9-1. Lineweaver-Burk plot of ABCC1 inhibition by β -cholanic acid. Panel 1 shows the optimization experiment to determine the time point at which uptake could be measured within the linear range. Panel 2 shows optimization data to determine the minimal range of accurately detectable levels of radio labeled doxorubicin. Panel 3 data shows lineweaver Burk analysis exhibiting convergence of two lines on the same x-intercept indicating a non-competitive inhibition on ABCC1 by β -cholanic acid. 4 highest concentration points are average of 3 trial, 6 lowest concentrations of dox are average of 5 trials. Linear regression analysis revealed that β -cholanic acid caused a small increase in the V_{\max} of drug uptake from 5.5×10^{-9} mMoles/h to 7.7×10^{-9} mMoles/h and a small decrease in K_m from 1.1×10^{-6} mM to 9.2×10^{-7} mM. These differences were further compared by using graphpad prims for lineweaver-Burk analysis as well as michaelis-menton kinetics with an n of 5 in each experiment and were found to be significant at $P < 0.05$.

Figure 9-2 Optimization of drug uptake measures for fluorescence assay

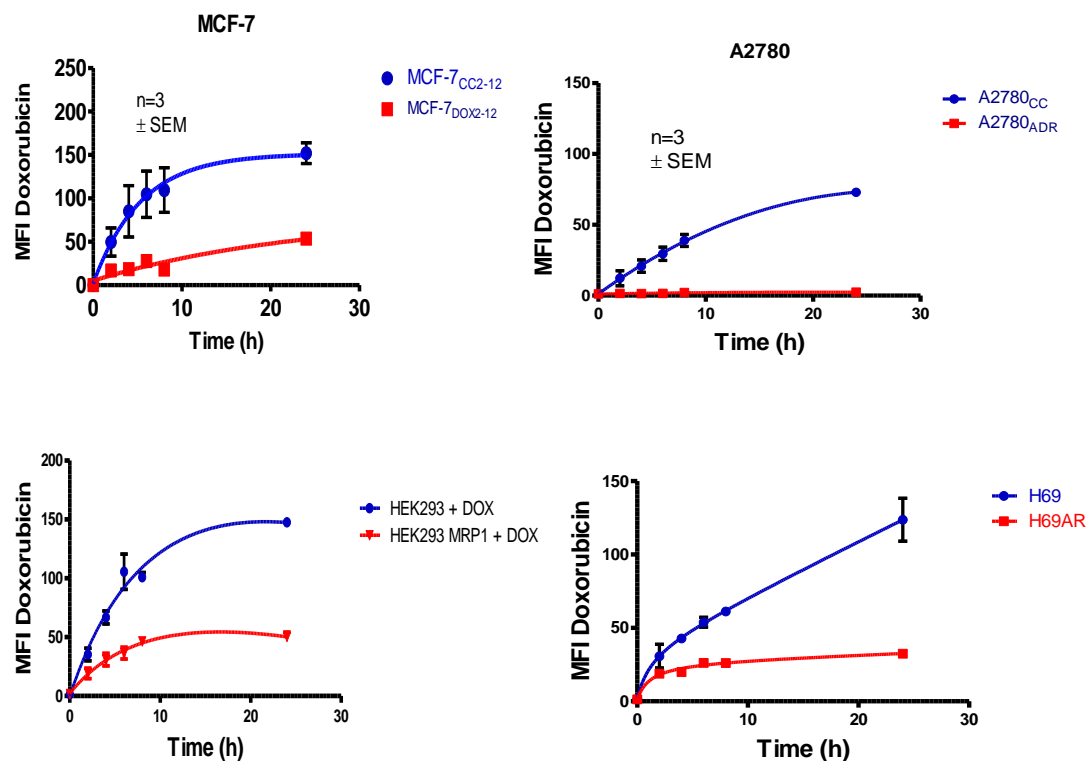


Figure 9-2 Optimization for measure of uptake of doxorubicin. Cells were treated with 2 μ M doxorubicin for varied times as indicated. Cells were then washed and lifted with trypsin as described in the methods section. Fluorescence intensities were recorded to determine the time at which optimal differences in uptake could be accurately measured.